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Cytoskeletal regulation of the L-arginine/NO pathway in pulmonary artery endothelial cells

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Zharikov, Sergey I., Alla A. Sigova, Sifeng Chen, Michael R. Bubb, and Edward R. Block. Cytoskeletal regulation of the L-arginine/NO pathway in pulmonary artery endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 280: L465–L473, 2001.—We investigated possible involvement of the actin cytoskeleton in the regulation of the L-arginine/nitric oxide (NO) pathway in pulmonary artery endothelial cells (PAEC). We exposed cultured PAEC to swinholide A (Swinh), which severs actin microfilaments, or jasplakinolide (Jasp), which stabilizes actin filaments and promotes actin polymerization, or both. After treatment, the state of the actin cytoskeleton, L-arginine uptake mediated by the cationic amino acid transporter-1 (CAT-1), Ca^{2+}/calmodulin-dependent (endothelial) NO synthase (eNOS) activity and content, and NO production were examined. Jasp (50–100 nM, 2 h treatment) induced a reversible activation of L-[^{3}H]arginine uptake by PAEC, whereas Swinh (10–50 nM) decreased L-[^{3}H]arginine uptake. The two drugs could abrogate the effect of each other on L-[^{3}H]arginine uptake. The effects of both drugs on L-[^{3}H]arginine transport were not related to changes in expression of CAT-1 transporters. Swinh (50 nM, 2 h) and Jasp (100 nM, 2 h) did not change eNOS activities and contents in PAEC. Detection of NO in PAEC by the fluorescent probe 4,5-diaminofluorescein diacetate showed that Swinh (50 nM) decreased and Jasp (100 nM) increased NO production by PAEC. The stimulatory effect of Jasp on NO production was dependent on the availability of extracellular L-arginine. Our results indicate that the state of actin microfilaments in PAEC regulates L-arginine transport and that this regulation can affect NO production by PAEC.

L-arginine transporter; nitric oxide production; actin cytoskeleton; pulmonary endothelial cells; swinholide; jasplakinolide

PULMONARY ENDOTHELIAL CELLS are a rich source of nitric oxide (NO), a nitrogen-centered free radical with multiple and unique physiological bioregulatory activities (36). Pulmonary endothelial cells generate NO from L-arginine via the catalytic action of a NADPH-requiring, Ca^{2+}/calmodulin-dependent (endothelial) NO synthase (eNOS) that is a peripheral membrane-associated protein (42). More recent studies have shown that eNOS, when appropriately acylated, targets to caveolae, specific cholesterol- and sphingolipid-rich microdomains of the plasma membrane (20, 47). The targeting of eNOS to caveolae in endothelial cells is promoted, in part, by direct interaction of the enzyme with the caveolar structural protein caveolin-1. There are data suggesting that the caveolar localization of eNOS in endothelial cells is critical for optimal NO production in stimulated and nonstimulated conditions (18, 30).

The activity of eNOS is not the only determinant controlling NO production by endothelial cells. Recent reports indicate that NO production, especially production stimulated by bradykinin (5), acetylcholine (1), shear stress (40), and endotoxin (44), is dependent on the availability and delivery of extracellular L-arginine. In endothelial cells, the delivery of extracellular L-arginine is mediated by the Na^+-independent system y^+ carrier, which is responsible for 70–95% of L-arginine uptake in endothelial cells (22, 56). Three system y^+ cationic amino acid transporters (CAT-1, CAT-2B, CAT-3) have been cloned, and the characteristic features of these transporters have been described (see reviews in Refs. 12 and 17). In porcine pulmonary artery endothelial cells (PAEC), the vast majority of system y^+-mediated Na^+-independent L-arginine uptake is provided by the CAT-1 transporter (56). Recently, we reported that the constitutive CAT-1 transporter and eNOS can colocalize in the caveolae of porcine PAEC and can be coimmunoprecipitated, suggesting a protein-to-protein interaction between CAT-1 and eNOS in these cells (33). We suggested that formation of such a caveolar complex promotes optimal NO production through the directed delivery of substrate (i.e., L-arginine) to eNOS.

Caveolae are specialized microdomains of the plasma membrane defined by their unique morphology and by the presence of one or more of a family of novel integral membrane proteins known as caveolins (2). Caveolae have been shown to be enriched in signaling molecules such as cell surface receptors, receptor tyrosine kinases, and G proteins (see reviews in Refs. 28 and 46). There are several reports demonstrating that

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plasmalemmal caveolae are closely related to cellular actin filaments (19, 29, 48). Recently, we reported that the CAT-1 L-arginine transporter interacts with the actin-binding protein fodrin and that proteolysis of fodrin can affect CAT-1 transport activity in PAEC (57). Therefore, it is tempting to speculate that the actin cytoskeleton may be involved in regulating caveolar function and, in particular, that changes in the organization of actin filaments may alter CAT-1-mediated L-arginine transport, the activity of eNOS, and/or NO production by endothelial cells. To test this, we exposed cultured porcine PAEC to agents with specific actin-binding activities and examined the state of the actin cytoskeleton (stress fibers), CAT-1-mediated L-arginine transport, eNOS activity, and NO production. Among the specific agents capable of affecting the actin cytoskeleton, we focused our study on two that easily penetrate cellular membranes and have opposite effects on actin microfilaments: swinholide A (Swinh) and jasplakinolide (Jasp) (50). Swinh is a microfilament-disrupting marine toxin that stabilizes actin dimers and severs actin filaments (7). Jasp binds to and stabilizes actin microfilaments and can promote actin polymerization (6, 8). Our results indicate that L-arginine transport in PAEC is regulated by the state of actin microfilaments and that changes in CAT-1 transport activity are predominantly responsible for changes in NO production by PAEC exposed to Swinh or Jasp.

MATERIALS AND METHODS

Reagents. We used commercial and noncommercial sources for Swinh and Jasp. Commercial Jasp was obtained from Molecular Probes (Eugene, OR). Commercial Swinh was obtained from Alexis Biochemicals. The commercially available agents induced the same changes in our experiments as the natural products isolated from marine sponges. Misakinolide A, latrunculin A and B, and halichondramide A were kindly provided by Dr. Michael R. Bubb. All agents were stored in methanol at −20°C and were diluted into medium immediately before use. The final methanol concentration in medium did not exceed 1%. All agents readily enter living mammalian cells when added to culture medium (50). L-[^{3}H]arginine was obtained from Amersham (Arlington Heights, IL). Mouse anti-eNOS polyclonal antibody was obtained from Transduction Laboratories (Lexington, KY). Fluorescence probe 4,5-diaminofluorescein diacetate (DAF-2DA) used for NO detection was purchased from Calbiochem (San Diego, CA). Peptide corresponding to the fourth extracellular domain of the human CAT-1 transporter (Y-F-G-V-S-A-A-L-T-L-M-M-P-Y-F-C-L-D-K-D-T-P-L-P-D-A-F-K-H-V-G-W-G) was synthesized by the Protein Core Facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida. Rabbit polyclonal antibodies to this peptide were prepared and characterized as previously reported by us (54).

Tissue culture. Endothelial cells were isolated by collagenase treatment of the main pulmonary artery of 6- to 7-mo-old pigs and were cultured and characterized as previously reported (4). Third- to fifth-passage cells in monolayer culture were maintained in RPMI 1640 medium containing 4% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin, and 1.5 μg/ml Fungizone) and were used 2 or 3 days after confluence.

Analysis of the actin microfilament system of PAEC. For the fluorescence microscopy analysis of microfilaments, PAEC were grown to near confluence on Mat Tech chamber slides (Mat Tech, Ashland, MA). Swinh and Jasp were added to the culture medium at final concentrations of 50–100 nM, and the cells were examined after a 2-h incubation. For F-actin staining, treated and untreated PAEC were washed twice in phosphate-buffered saline (PBS), permeabilized with 0.05% Triton X-100 for 3 min at room temperature, and then fixed in 4% formaldehyde in PBS for 10 min at room temperature, washed three times with PBS, and blocked in 1% BSA and 50 mM L-lysine in PBS for 1 h at 4°C. Labeling of F-actin with fluorescein-phalloidin was done per the manufacturer's directions (Molecular Probes, Eugene, OR). Images were digitally recorded using a Zeiss Axiovert 100M microscope with a Zeiss LSM5 confocal attachment.

Measurement of CAT-1-mediated Na^+-independent L-arginine transport by PAEC. Transport of radiolabeled L-arginine by PAEC was measured as reported by our group (57, 58). In brief, to remove residual culture medium and extracellular Na^+, cells grown in 24-well plates were washed with a buffer of the following composition (in mM): 140 LiCl, 5 KCl, 2 Na_2HPO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 11 glucose, and 10 HEPES-Tris (pH 7.4, LiCl-Dulbecco solution). Transport assays were initiated by the addition of the same buffer containing 50 μM unlabeled L-arginine plus L-[^{3}H]arginine (5 μCi/ml), and 30 s later, transport was stopped by washing the cells four times with ice-cold buffer. After solubilization of the cells in 0.2% SDS, aliquots were added to scintillation fluid, and radioactivity was quantitated by liquid scintillation spectrometry. All measurements of CAT-1-mediated Na^+-independent L-arginine uptake were corrected by subtracting the nonspecific component of uptake.

Determination of eNOS activity in PAEC. eNOS activity in intact cells was determined by monitoring the conversion of L-[^{3}H]arginine into L-[^{3}H]citrulline using methods previously described (16, 43). Control cells and cells incubated for 2 h with 50 nM Swinh, 100 nM Jasp, or both drugs together were washed once in 1 ml of warmed LiCl-Dulbecco solution and then incubated in 0.5 ml of LiCl-Dulbecco solution containing L-[^{3}H]arginine (5 μCi/ml) for 15 min. After the 15-min incubation, PAEC were washed three times with 2 ml of ice-cold LiCl-Dulbecco solution containing 5 mM EDTA and were then lysed in 1 ml of 10 mM HCl containing 0.1% SDS. Two aliquots of lysates (50 μl each) were removed for measurements of protein content and radioactivity incorporated during the incubation with labeled L-arginine. To the remaining sample (0.9 ml), 0.1 ml of 0.2 M sodium acetate buffer, pH 13.0, containing 10 mM L-citrulline was added, and the sample was applied to a column of Dowex AG50WX 8 (H^+ form) added as a 1:1 slurry in H_2O. The effluents were collected in counting vials and, subsequent to elution of residual L-[^{3}H]citrulline with 1 ml of H_2O, 10 ml of scintillation fluid were added for determination of eluted radioactivity. eNOS activity is expressed as percent conversion of incorporated L-[^{3}H]arginine into L-[^{3}H]citrulline. Protein determinations were made by the method of Lowry et al. (31).

Determination of eNOS activity in the total membrane fraction from PAEC. Control and treated PAEC were scraped and homogenized in *buffer A* (50 mM Tris·HCl, pH 7.4, containing 0.1 mM each EDTA and EGTA, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin). The homogenates were centrifuged at 100,000 *g* for 60 min at 4°C, and the total membrane fraction pellet was resuspended in *buffer B* (*buffer A* plus 2.5 mM $CaCl_2$) and used for eNOS activity

determinations by monitoring the formation of L-[^{3}H]citrulline from L-[^{3}H]arginine (38). Membranes (100–120 μg of protein) were incubated (total volume 0.4 ml) in *buffer A* with the addition of 1 mM NADPH, 100 nM calmodulin, 10 μM tetrahydrobiopterin, and 5 μM combined L-arginine and purified L-[^{3}H]arginine for 30 min at 37°C. The measurement of L-[^{3}H]citrulline formation was performed as described by us (38).

Western blot analysis of eNOS and CAT-1 contents in cell lysates. PAEC were washed twice with ice-cold PBS without Ca^{2+} or Mg^{2+} and scraped in lysis buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 0.4% deoxycholate, and 60 mM octylglucoside) containing protease inhibitor cocktail (Calbiochem). The cell extracts were centrifuged at 100,000 *g* for 30 min, and the supernatants were kept at −80°C until use. Samples (15–20 μg of protein) were separated by SDS-PAGE (7.5% acrylamide) and transferred to nitrocellulose. The nitrocellulose membranes were incubated in blocking solution (10 mM Tris, 5% nonfat dry milk, and 100 mM NaCl, pH 7.5) for at least 1 h and then hybridized overnight at 4°C with a 1:1,000 dilution of polyclonal anti-eNOS antibody or a 1:800 dilution of a polyclonal anti-CAT-1 antibody. Alkaline phosphatase-conjugated secondary antibodies (1:3,000 dilution; Bio-Rad) were used to visualize bound primary antibodies with chemiluminescence substrate (Immuno-Star; Bio-Rad). The density of the bands was quantitated using a Fluor-S MultiImager system (Bio-Rad).

Analysis of NO production by PAEC. NO production was measured in real time using DAF-2DA, a specific fluorescence probe for NO detection (26). DAF-2DA is a membrane-permeable dye that is hydrolyzed inside the cells by cytosolic esterases releasing DAF-2. In the presence of NO, DAF-2 converts into a fluorescent product, DAF-2 triazole, which can be detected (37). For NO detection, PAEC were grown on 96-well, clear-bottom plates. Before the experiment, cells were washed once and incubated in 0.1 ml of Krebs-Henseleit solution [in mM: 132 NaCl, 3.9 KCl, 2.8 $CaCl_2$, 1.5 KH_2PO_4, 1.2 $MgCl_2$, 10 HEPES (pH 7.4), and 5.5 glucose] containing 20 μM DAF-2DA for 1 h at 37°C. After the incubation, cells were washed and incubated in 0.1 ml of Krebs-Henseleit solution containing 50 μM L-arginine and various concentrations of Swinh or Jasp. All measurements of specific DAF-2DA fluorescence were corrected by subtracting the nonspecific fluorescence in wells without addition of DAF-2DA and in wells without cells. Fluorescence was measured using an FL600 microplate fluorescence reader (Bio-TEK Instrument, Winooski, VT) every 5 min (excitation wavelength = 488 nm and emission wavelength = 510 nm).

Data analysis. In each experiment, treated and control PAEC were matched for cell line, age, number of passages, and number of days postconfluence to avoid variations in tissue culture factors that can influence the measurements of L-[^{3}H]arginine uptake, eNOS activity, and NO production. Data are expressed as means ± SD. Comparisons between values were made using an unpaired, two-tailed Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effects of Swinh and Jasp on L-arginine uptake and organization of the actin cytoskeleton in PAEC. To investigate whether changes in organization of the actin cytoskeleton affect L-arginine transport, cultured PAEC were exposed to several marine natural products that have been reported to target the actin cytoskeleton and to change its organization (50). Special attention was paid to Swinh and Jasp because of their opposing effects on microfilament structure.

PAEC were exposed to Swinh or Jasp for 0.5–12 h (Fig. 1*A*). Exposure to 10 nM Swinh had no significant effect on L-[^{3}H]arginine uptake, whereas exposure to 20 nM and 50 nM Swinh resulted in significant dose-dependent and sustained decreases in L-[^{3}H]arginine uptake. In contrast, exposure of PAEC to the microfilament-stabilizing agent Jasp (50 nM and 100 nM) resulted in significant increases in L-[^{3}H]arginine uptake, which were maximal after 2 h of incubation. No evidence of cell toxicity, assessed by cell number, cell protein content, and lactate dehydrogenase release, was observed with the concentrations of Swinh or Jasp used in our experiments. The decrease in L-arginine transport observed in cells incubated with Swinh was also observed with other marine agents known to disrupt the actin microfilament system, including misakinolide A, which sequesters unpolymerized actin and caps the barbed (growing) ends, latrunculin A and B, which sequester actin monomers, and halichon dramide, which severs microfilaments (Table 1).

Because Swinh disrupts actin microfilaments and Jasp stabilizes preexisting actin microfilaments, we examined whether Jasp might block the Swinh-induced decrease in L-arginine transport and/or whether Swinh might prevent the Jasp-induced increase in L-arginine transport. To do this, PAEC were incubated with Swinh alone, Jasp alone, or Swinh plus Jasp for 2 h. As expected, Swinh decreased uptake, Jasp increased uptake, and the coincubation resulted in no net effect on L-arginine transport (Fig. 1*B*).

To assess whether the effects of Swinh or Jasp were reversible, PAEC were incubated with Swinh or Jasp for 2 h, washed, and then returned to fresh culture medium without agents for 2 h for recovery. As shown in Fig. 1*C*, the effects of Swinh and Jasp on L-arginine transport by PAEC were reversible after removal of the cytoskeleton-active agents from the culture medium.

Western blot analysis with antibodies directed against the CAT-1 L-arginine transporter showed that a 2-h treatment with Swinh (50 nM) or Jasp (100 nM) did not change the expression of CAT-1 transporter in PAEC (Fig. 1*D*). These results are consistent with posttranslational effects of Swinh and Jasp on CAT-1-mediated L-arginine transport by porcine PAEC.

Control PAEC exhibited a network of cytoplasmic actin stress fibers and an incomplete rim of cortical actin filaments that were relatively substantial (Fig. 2*A*). Jasp (100 nM) treatment resulted in only subtle changes. The cortical rim of filaments was mostly unchanged. Stress fibers were thicker but probably not more numerous (Fig. 2*B*). As with the control cells, there was substantial intracellular variation, and areas of confluency, in general, had better spread cells with more abundant stress fibers. In some cells, there existed small amounts of perinuclear actin aggregates as has been previously described in other cultured cells (Fig. 2*C*) (8). Swinh (50 nM) treatment resulted in nearly complete loss of stress fibers, with some remain-

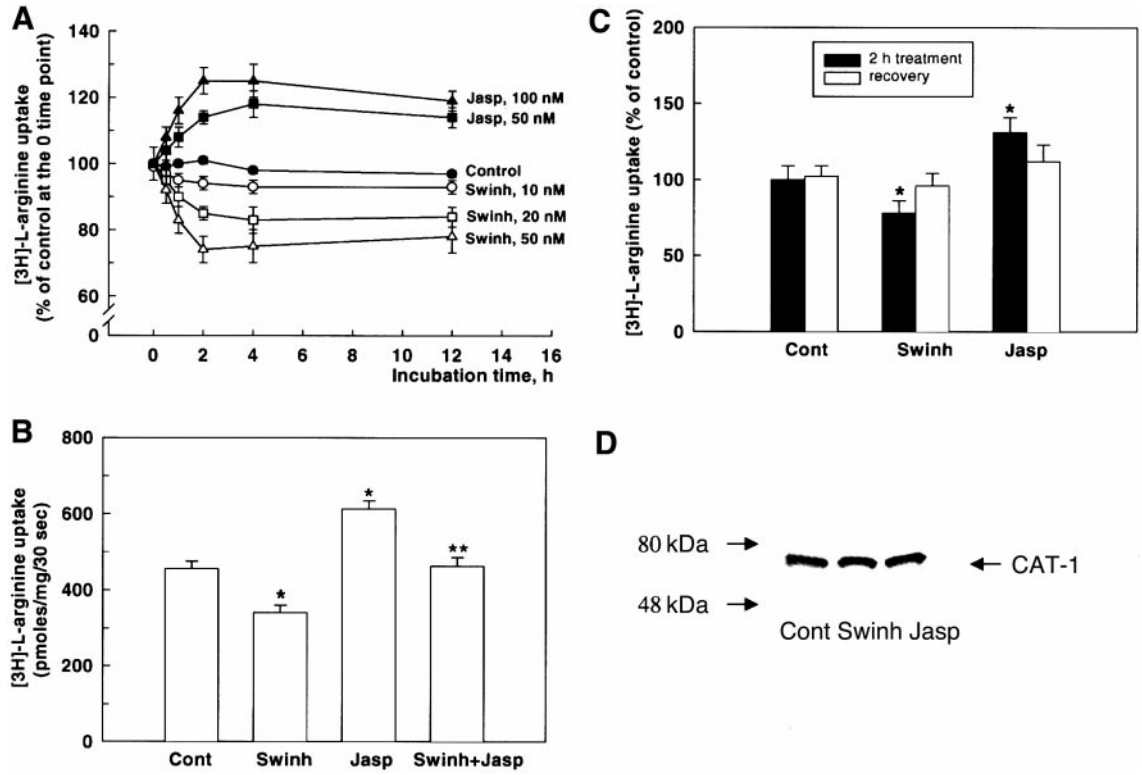

Fig. 1. Effects of swinholide A (Swinh) and jasplakinolide (Jasp) on L-arginine uptake by pulmonary artery endothelial cells (PAEC). *A*: effect of treatment time and concentrations of Swinh and Jasp on L-arginine uptake. PAEC grown in 24-well cluster trays were treated with different Swinh and Jasp concentrations for 0.5–12 h at 37°C. After the treatment, PAEC were washed with LiCl-Dulbecco solution, and cationic amino acid transporter (CAT-1)-mediated Na^+-independent uptake of 50 μM L-[^{3}H]arginine by PAEC was measured. Results are from 6 separate experiments with 8 replicates in each experiment and are expressed as means ± SD in percent of L-[^{3}H]arginine uptake by untreated control PAEC at the 0 time point. *B*: effect of simultaneous incubation with Swinh and Jasp on L-[^{3}H]arginine uptake by PAEC. The cells were treated with 50 nM Swinh alone, 100 nM Jasp alone, or simultaneously with 50 nM Swinh and 100 nM Jasp for 2 h. Immediately after exposure, the media were discarded, cells were washed with LiCl-Dulbecco solution, and Na^+-independent transport of 50 μM L-[^{3}H]arginine was measured. Results are the means ± SD of 4–6 separate experiments with 8 replicates in each experiment. *$P < 0.05$ vs. control. **$P < 0.05$ vs. Swinh alone or Jasp alone and >0.05 vs. control. *C*: reversibility of the effects of 50 nM Swinh and 100 nM Jasp on L-[^{3}H]arginine transport in PAEC. Cells grown in 24-well cluster trays were incubated with the drugs for 2 h at 37°C. Immediately after exposure, the media were discarded, and cells in each well were washed twice for 30 min in RPMI 1640 at 37°C and then incubated in fresh RPMI 1640 for an additional 2 h at 37°C before the uptake experiment. Control cells were incubated in RPMI 1640 alone and underwent washing and return of fresh RPMI 1640. Results are the mean values of 3 separate experiments with 8 replicates in each experiment and are expressed as means ± SD in percent of L-[^{3}H]arginine uptake by untreated control PAEC. *$P < 0.05$ vs. control. *D*: Western blot analysis of CAT-1 transporter contents in cell lysates obtained from control cells and cells treated with Swinh (50 nM, 2 h) or Jasp (100 nM, 2 h). CAT-1 bands from a typical experiment are shown. Quantitative densitometric analysis of CAT-1 contents from 3 separate experiments did not reveal significant differences between control and Swinh- or Jasp-treated cells.

ing cortical actin (Fig. 2*D*). Swinh-treated PAEC were contracted and less adherent than control PAEC.

Effects of Swinh and Jasp on eNOS activity and content in PAEC. The measurements of L-[^{3}H]citrulline formation from L-[^{3}H]arginine by intact PAEC showed that Swinh (50 nM, 2-h treatment) decreased and Jasp (100 nM, 2-h treatment) increased the absolute amount of L-[^{3}H]citrulline produced by PAEC compared with control cells. However, the calculation of the fraction of L-[^{3}H]arginine taken up by cells that was converted to L-[^{3}H]citrulline showed that the velocity of L-[^{3}H]citrulline formation, which is a measure of eNOS activity, was comparable in control cells and cells treated with one or both of the cytoskeleton-active agents (Fig. 3*A*). The measurements of eNOS activity in the total membrane fractions isolated from PAEC also did not show significant differences in eNOS activity between control PAEC (10.0 ± 0.6 pmol L-citrulline·mg protein^{-1}·min^{-1}) and PAEC incubated for 2 h with 50 nM Swinh (8.4 ± 0.7 pmol L-citrulline·mg protein^{-1}·min^{-1}) or 100 nM Jasp (8.3 ± 1.0 pmol L-citrulline·mg protein^{-1}·min^{-1}). Finally, Western blot analysis of eNOS contents in cell lysates demonstrated that Swinh (50 nM, 2-h treatment) and Jasp (100 nM, 2-h treatment) did not change the amount of eNOS per microgram of cell protein (Fig. 3*B*). Taken together, these data demonstrate that a 2-h treatment with Swinh (50 nM) or Jasp (100 nM) did not

Table 1. *Effect of marine toxins on L-[^{3}H]arginine uptake by PAEC*

Marine Toxin	Mechanism of Microfilament Disruption	%Inhibition of L-[^{3}H]arginine Uptake	*n*
Misakinolide A (10 nM, 24-h treatment)	Sequestering and capping of microfilaments	34	3
Latrunculin A (100 nM, 24-h treatment)	Actin monomer sequestering	31	4
Latrunculin B (100 nM, 24-h treatment)	Actin monomer sequestering	17	5
Halichondramide A (10 nM, 24-h treatment)	Microfilament severing	56	3

Values are means; *n*, no. of experiments. Pulmonary artery endothelial cells (PAEC) grown in 24-well cluster trays were exposed to toxins dissolved in culture media at the indicated concentrations for 24 h. After the treatment, L-[^{3}H]arginine transport in PAEC was measured. All changes in L-arginine uptake by PAEC were significant, with $P < 0.05$ vs. untreated control PAEC.

significantly change eNOS expression or eNOS activity in PAEC.

Effects of Swinh and Jasp on NO production by PAEC. Both Swinh and Jasp changed NO production by PAEC. As shown in Fig. 4*A*, Swinh (50–200 nM) induced dose-dependent decreases in NO production in cultured PAEC compared with control PAEC. The total amounts of NO produced by the cultured PAEC after a 5-h incubation, as detected by the fluorescent indicator DAF-2DA, were 40 and 370% less after treatment with Swinh in concentrations of 50 and 200 nM, respectively, than in control cells for the same period of time. Jasp resulted in a dose-dependent increase in NO production by cultured PAEC (Fig. 4*A*). PAEC treated with 100 nM Jasp for 5 h produced about three times more NO than untreated cells.

As shown in Fig. 4*B*, the magnitude of the stimulatory effect of Jasp on NO production was dependent on the presence of extracellular L-arginine. Jasp (100 nM) was able to stimulate NO production by PAEC in the absence of extracellular L-arginine, but its stimulatory effect on NO production was significantly lower than in the presence of 50 μM extracellular L-arginine.

DISCUSSION

Endothelial cells contain a well-developed actin microfilament cytoskeleton that has been shown to affect endothelial cell structure and function (21). Several reports suggest that structural changes in the endothelial cytoskeleton provide a transduction pathway between shear stress and the synthesis of NO (23, 25). Participation of the actin cytoskeleton in the regulation of NO production by PAEC may be mediated through effects on the availability of L-arginine, on eNOS activity, or on both. The current study was designed to determine whether changes in the actin cytoskeleton affect L-arginine uptake or eNOS activity or both, and if so, whether these effects would influence NO production. To change actin microfilament structure in PAEC, we chose two marine sponge toxins, Swinh and Jasp, possessing opposite effects on the microfilament structure (6–8). The advantages of these toxins are that they are readily cell permeable, they can be used for modification of the cytoskeleton without any additional cell treatments (50), and they can be used in endothelial cells in dosages that modulate the cytoskeleton (Fig. 2) but do not cause cytotoxicity.

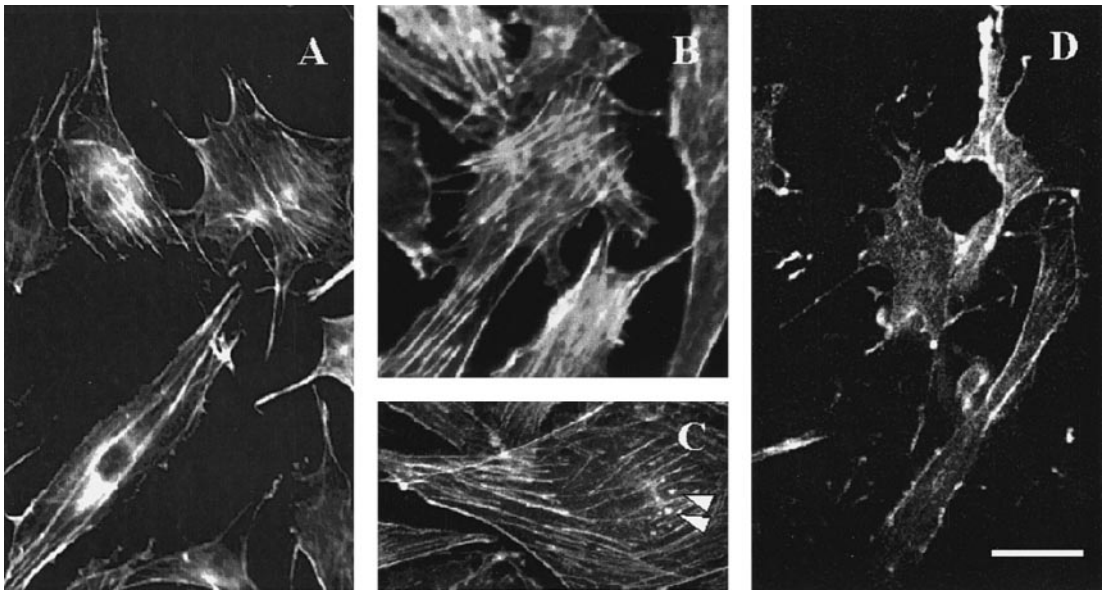

Fig. 2. Effects of Swinh and Jasp on cell morphology and actin filament distribution. *A*: control cells. *B* and *C*: Jasp-treated PAEC with perinuclear actin aggregates (arrowheads). *D*: Swinh-treated PAEC.

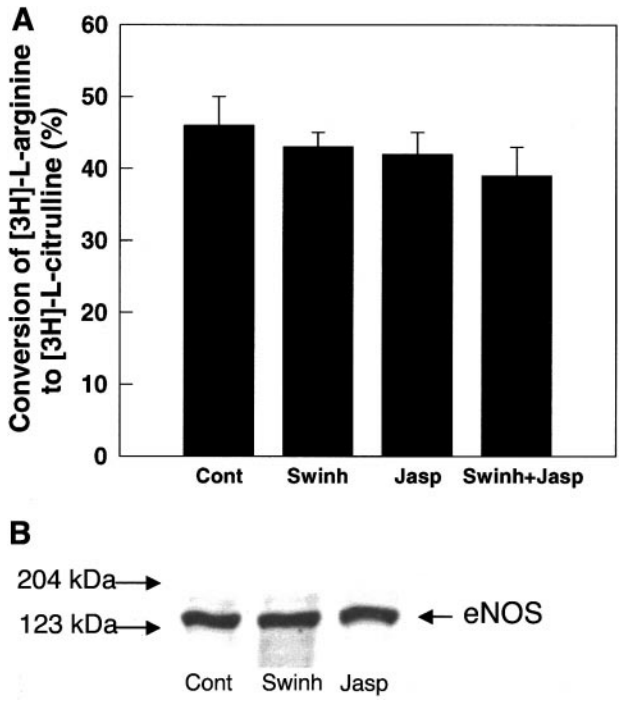

Fig. 3. Effects of Swinh and Jasp on Ca^{2+}/calmodulin-dependent (endothelial) NO synthase (eNOS) activity and content in PAEC. *A*: effect of Swinh and Jasp on the conversion of incorporated L-[^{3}H]arginine to L-[^{3}H]citrulline in whole cells. PAEC grown in 24-well plates were treated with Swinh (50 nM), Jasp (100 nM), or both agents for 2 h. After the treatment, eNOS activity, expressed as percent conversion of L-[^{3}H]arginine into L-[^{3}H]citrulline, was determined as described in MATERIALS AND METHODS. *B*: Western blot for eNOS in cellular lysates obtained from control PAEC and PAEC treated with Swinh (50 nM) or Jasp (100 nM) for 2 h. Western blot analysis of eNOS was performed as described in MATERIALS AND METHODS. Quantitative densitometric analysis of eNOS bands did not reveal significant differences in eNOS contents between control cells and cells treated with Swinh or Jasp in 4 separate experiments.

Our results demonstrate that changes in actin microfilament structure affect CAT-1-mediated Na^+-independent L-arginine transport activity in porcine PAEC. Stabilization of actin microfilaments by Jasp increased L-arginine uptake by PAEC, whereas disruption of microfilaments by Swinh and various other agents inhibited L-arginine transport. The effects of Swinh and Jasp were reversible, and both agents could abolish the effects of each other. These results indicate that the changes in L-arginine uptake induced by the action of Swinh and Jasp are directly connected to modifications of the actin cytoskeleton in PAEC. Because the changes in L-arginine uptake were not accompanied by changes in CAT-1 transporter expression in the PAEC, they are likely due to actin microfilament-induced changes in existing transporters or their environment.

There are a number of observations that the activity of membrane proteins can be regulated by the actin cytoskeleton (34). Dependence on the state of actin microfilaments has been reported for Na^+ channel (11) and Na^+-K^+-ATPase (10) activities in epithelial cells, for Ca^{2+} transport in fibroblasts (55), for the activity of the cystic fibrosis transmembrane conductance regulator (CFTR) in mouse adenocarcinoma cells and human epithelial cells (41), and for Cl^- secretion in intestinal cells (32). The precise mechanisms responsible for cytoskeletal regulation of the activity of integral membrane proteins have not been worked out to date. Several investigators have suggested that a regulatory role for actin microfilaments may be mediated through changes in the secondary structure of membrane proteins as a result of direct binding to the cytoskeleton (10, 41) because distinct domains of some membrane proteins, e.g., CFTR and Na^+-K^+-ATPase, may represent relevant actin-binding domains. Microfilament regulation of membrane proteins may also be mediated by various actin-binding proteins. Recently, we reported that the CAT-1 L-arginine transporter interacts with the actin-binding protein fodrin in PAEC and that degradation of fodrin affects L-arginine transport activity in these cells (57). Therefore, it is possible that Swinh and Jasp modulate the stability of a "CAT-1-fodrin-actin microfilament" complex that affects transporter activity directly or affects the localization, and in turn function, of the CAT-1 transporter in specific membrane domains. Because the fodrin-based membrane skeleton is a submembranous structure that binds a subset of membrane proteins (3), the cytoskeleton changes induced by Jasp and Swinh can enhance or weaken, respectively, the interaction of CAT-1 with proteins that serve to regulate the L-arginine transporter system.

In contrast to L-arginine transport, neither eNOS activity nor eNOS protein content was altered by incubation with Swinh or Jasp for 2 h. Unlike the CAT-1 transporter, which is an integral membrane protein penetrating the plasma membrane, eNOS is a peripheral membrane protein that is targeted to specific cellular domains, including Golgi and plasmalemmal caveolae. Targeting of eNOS to plasmalemmal caveolae is dependent on the myristoylation (47) and palmitoylation (20) of eNOS. Sessa and colleagues (45) reported that eNOS association with Golgi membranes is also acylation dependent. Inhibition of acylation and mutation of the palmitoylation sites attenuate Golgi and caveolae targeting of eNOS (20, 45) and markedly reduce the stimulated production of NO (30). The biological function of Golgi-localized eNOS and the relationship between caveolar and Golgi pools of eNOS are not fully understood.

Several studies indicate that translocation of eNOS between the Golgi and plasmalemmal caveolae is a cytoskeleton-dependent process. For example, it has been shown that bradykinin-induced translocation of eNOS in endothelial cells is accompanied by rearrangement of F-actin (35, 53) and by translocation of a dimeric actin-cross-linking protein, filamin (52, 53). Moreover, blocking filamin phosphorylation prevents bradykinin-induced F-actin rearrangement and filamin and eNOS translocation in these cells (53). However, Venema and colleagues (51) reported that bradykinin-induced translocation of eNOS from Triton X-100-soluble membranes to the cytoskeleton produced

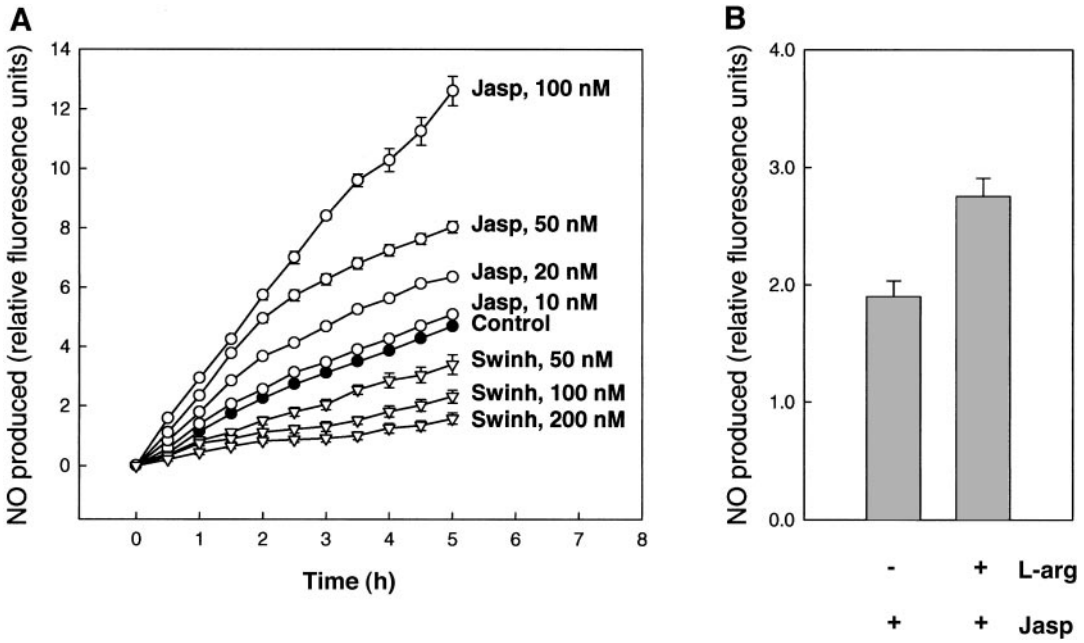

Fig. 4. Effects of Swinh and Jasp on NO production by PAEC. *A*: real-time measurements of NO produced by control cells and cells treated with Swinh (50–200 nM) or Jasp (10–100 nM). NO detection was carried out as described in MATERIALS AND METHODS. Data of a typical experiment with 8 replicates are shown. *B*: dependence of the stimulatory effect of Jasp on NO production on the presence of extracellular L-arginine. Shortly after the addition of fluorescent probe for detection of NO (5-s delay), Jasp (100 nM) was added to the wells containing cultured PAEC with and without 50 μM L-arginine (L-arg) in the medium. After incubation for 1 h, the intensity fluorescence was detected as described in MATERIALS AND METHODS. Results are means ± SD of 4 independent measurements.

no detectable change in eNOS activity of cell lysates. Furthermore, time-dependent changes in the eNOS activity of the Triton X-100-soluble (presumably Golgi-localized membranes) and Triton X-100-insoluble (presumably caveolar) fractions were proportional to the changes in the amount of the enzyme detected on immunoblots. The authors concluded that association of eNOS with cytoskeletal elements did not function as a mechanism for regulation of enzyme activity. This conclusion is in good agreement with our results showing that 2-h incubations with Swinh and Jasp do not alter eNOS activity or expression in cultured PAEC. However, our results do not preclude the possibility that longer incubations with these agents may result in alterations in eNOS activity or expression.

Our results also show that Swinh- and Jasp-induced rearrangements in the actin cytoskeleton alter NO production by PAEC. The changes in NO production paralleled the changes in L-arginine transport in these cells, i.e., stabilization of actin microfilaments increased NO production and L-arginine uptake, whereas the disruption of microfilaments by Swinh decreased NO production and L-arginine transport in a concentration-dependent manner. All of these changes occurred without any change in eNOS activity in the PAEC. These results suggest that NO production by PAEC is dependent on L-arginine transport. The dependence of NO production by vascular endothelial cells on extracellular L-arginine has been observed in a number of in vitro and in vivo studies (1, 14, 15, 39). These observations, i.e., that extracellular L-arginine administration seems to drive NO production even when intracellular levels of L-arginine are available in excess, have been referred to as the "arginine paradox" (27). Recently, we reported (33) that CAT-1 L-arginine transporters interact with and colocalize with eNOS molecules, forming a functional "CAT-1-eNOS" complex in the caveolae of cultured PAEC that can provide directed delivery of extracellular L-arginine to eNOS for NO synthesis. The parallel changes in L-arginine transport and NO production during the toxin-induced rearrangements of the actin cytoskeleton observed in our experiments fit well with the concept of such a complex.

The existence of CAT-1-eNOS complexes in PAEC is also supported by our data that removal of extracelluar L-arginine from the medium affected Jasp-stimulated NO production. Thus the stimulatory effect of Jasp on NO production was significantly dependent on the presence of extracellular L-arginine (Fig. 4*B*). However, Jasp could stimulate NO production after the removal of extracellular L-arginine from the medium to achieve an "L-arginine-free" medium. Recently, Closs et al. (13) suggested the existence of two pools of intracellular L-arginine in endothelial cells, one of which ("not freely exchangeable with extracellular space") might be a membrane-encircled compartment that can be used by eNOS for NO synthesis. If such a pool exists in porcine PAEC, then the stimulatory effect of Jasp on NO production in our cells in L-arginine-free medium may be

related to an effect of Jasp on the accessibility of L-arginine from this intracellular pool to eNOS. However, there might also be another explanation for the stimulatory effect of Jasp on NO production after removal of extracellular L-arginine from the medium. Accumulation of L-arginine by cells depends on membrane potential (9, 24, 49, 58) and, at the steady state, there is an equilibrium between the inward directed, transporter-mediated flux of L-arginine driven by membrane potential and the outward directed efflux supported by an electrochemical L-arginine gradient (56). At the normal membrane potential (about −60 mV), the estimated ratio of intracellular to extracellular L-arginine in cultured PAEC should be 1:10. After removal of extracellular L-arginine, at the same membrane potential, a portion of intracellular L-arginine will be released from cells and a new steady-state equilibrium will be reestablished at new concentrations of intracellular and extracellular L-arginine. Quantitation of the medium concentration of L-arginine after changing an L-arginine-containing medium to an L-arginine-free medium by HPLC showed that, under the conditions of our experiments, extracellular (i.e., released) L-arginine concentration in the L-arginine-free medium was 7 ± 2 μM. This released L-arginine can be delivered by CAT-1 transporters to eNOS for NO production, and this delivery can be affected by Jasp. This may account for why Jasp is able to stimulate NO production in L-arginine-free medium.

In conclusion, our results indicate that the state of actin microfilaments in PAEC can regulate Na^+-independent CAT-1-mediated L-arginine transport and that this regulation affects NO production in these cells in the absence of a change in eNOS activity or content. The dependence of NO production by PAEC on CAT-1 transporter activity adds further support to the concept of CAT-1-mediated directed delivery of L-arginine to eNOS.

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REFERENCES

1. **Aisaka K, Gross S, Griffith OW, and Levi R.** L-Arginine availability determines the duration of acetylcholine-induced systemic vasodilation in vivo. *Biochem Biophys Res Commun* 163: 710–717, 1989.
2. **Anderson RGW.** The caveolae membrane system. *Annu Rev Biochem* 67: 199–225, 1998.
3. **Beck KA and Nelson WJ.** The spectrin-based membrane skeleton as a membrane protein-sorting machine. *Am J Physiol Cell Physiol* 270: C1263–C1270, 1996.
4. **Block ER, Herrera H, and Couch M.** Hypoxia inhibits L-arginine uptake by pulmonary artery endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 269: L574–L580, 1995.
5. **Bogle RG, Coade SB, Moncada S, Pearson JD, and Mann GE.** Bradykinin and ATP stimulate L-arginine uptake and nitric oxide release in vascular endothelial cells. *Biochem Biophys Res Commun* 180: 926–932, 1991.
6. **Bubb MR, Senderowicz AMJ, Sausville EA, Duncan KLK, and Korn ED.** Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. *J Biol Chem* 269: 14869–14871, 1994.
7. **Bubb MR, Spector I, Bershadsky AD, and Korn ED.** Swinholide is a microfilament disrupting marine toxin that stabilizes actin dimers and severs actin filaments. *J Biol Chem* 270: 3463–3466, 1995.
8. **Bubb MR, Spector I, Beyer BB, and Fosen KM.** Effects of jasplakinoloide on the kinetics of actin polymerization. An explanation for certain in vivo observations. *J Biol Chem* 275: 5163–5170, 2000.
9. **Bussolati O, Laris PC, Nucci FA, Dall'Asta V, Longo N, Guidotti GG, and Gazzola GC.** Dependence of L-arginine accumulation on membrane potential in cultured human fibroblasts. *Am J Physiol Cell Physiol* 253: C391–C397, 1987.
10. **Cantiello HF.** Actin filaments stimulate the Na^+-K^+-ATPase. *Am J Physiol Renal Fluid Electrolyte Physiol* 269: F637–F643, 1995.
11. **Cantiello HF, Stow JL, Prat AD, and Ausiello DA.** Actin filaments regulate epithelial Na^+ channel activity. *Am J Physiol Cell Physiol* 261: C882–C888, 1991.
12. **Closs EI.** CATs, a family of three distinct mammalian cationic amino acid transporters. *Amino Acids (Vienna)* 11: 193–208, 1996.
13. **Closs EI, Scheld J-S, Sharafi M, and Forstermann U.** Substrate supply for nitric-oxide synthase in macrophages and endothelial cells: role of cationic amino acid transporters. *Mol Pharmacol* 57: 68–74, 2000.
14. **Cooke JP, Andon NA, Girerd XJ, Hirsch AT, and Creager MA.** Arginine restores cholinergic relaxation of hypercholesterolemic rabbit thoracic aorta. *Circulation* 83: 1057–1062, 1991.
15. **Creager MA, Gallagher SJ, Girerd XJ, Coleman SM, Dzau VJ, and Cooke JP.** L-Arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. *J Clin Invest* 90: 1248–1253, 1992.
16. **Davda RK, Chandler LJ, Crews FT, and Guzman NJ.** Ethanol enhances the endothelial nitric oxide synthase response to agonists. *Hypertension* 21: 939–943, 1993.
17. **Deves R and Boyd CAR.** Transporters for cationic amino acids in animal cells: discovery, structure, and function. *Physiol Rev* 78: 487–545, 1998.
18. **Feron O, Saldana F, Michel JB, and Michel T.** The eNOS-caveolin regulatory cycle. *J Biol Chem* 273: 3125–3128, 1998.
19. **Fujimoto T, Miyawaki A, and Mikoshiba K.** Inositol 1,4,5-trisphosphate receptor-like protein in plasmalemmal caveolae is linked to actin filaments. *J Cell Sci* 108: 7–15, 1996.
20. **Garcia-Cardena G, Oh P, Liu J, Schnitzer JE, and Sessa WC.** Targeting of nitric oxide synthase to endothelial cell caveolae via palmitoylation: implication for nitric oxide signaling. *Proc Natl Acad Sci USA* 93: 6448–6453, 1996.
21. **Gottlieb AI, Langille BL, Wong MKK, and Kim DW.** Structure and function of the endothelial cytoskeleton. *Lab Invest* 65: 123–137, 1991.
22. **Green B, Pacitti AJ, and Souba WW.** Characterization of L-arginine transport by pulmonary artery endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 264: L351–L356, 1993.
23. **Hutcheson IR and Griffith TM.** Mechanotransduction through the endothelial cytoskeleton: mediation of flow- but not agonist-induced EDRF release. *Br J Pharmacol* 118: 720–726, 1996.
24. **Kavanaugh MP.** Voltage dependence of facilitated arginine flux mediated by the system y^+ basic amino acid transporter. *Biochemistry* 32: 5781–5785, 1993.
25. **Knudsen HL and Frangos JA.** Role of cytoskeleton in shear stress-induced endothelial nitric oxide production. *Am J Physiol Heart Circ Physiol* 273: H347–H355, 1997.
26. **Kojima H, Nakatsubo N, Kikuchi K, Kawahara S, Kirino Y, Nagoshi H, Hirata Y, and Nagano T.** Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Anal Chem* 70: 2446–2453, 1998.
27. **Kurz S and Harrison DG.** Insulin and the arginine paradox. *J Clin Invest* 99: 369–370, 1997.

28. **Lisanti MP, Scherer PE, Tang Z, and Sargaicomo M.** Caveolae, caveolin and caveolin-rich membrane domains: a signalling hypothesis. *Trends Cell Biol* 4: 231–235, 1994.
29. **Lisanti MP, Scherer PE, Vidugiriene J, Tang Z, Hermanowski-Vosatka A, Tu YH, Cook RF, and Sargiacomo M.** Characterization of caveolin-rich membrane domains isolated from an endothelial-rich source: implications for human disease. *J Cell Biol* 126: 111–126, 1994.
30. **Liu. J, Garcia-Cardena G, and Sessa WC.** Palmitoylation of endothelial nitric oxide synthase is necessary for optimal stimulated release of nitric oxide: implications for caveolae localization. *Biochemistry* 35: 13277–13281, 1996.
31. **Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ.** Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265–275, 1951.
32. **Matthews JB, Smith JA, and Hrnjez BJ.** Effects of F-actin stabilization or disassembly on epithelial Cl^- secretion and Na-K-2Cl cotransport. *Am J Physiol Cell Physiol* 272: C254–C262, 1997.
33. **McDonald KK, Zharikov S, Block ER, and Kilberg MS.** A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric oxide synthase may explain the "arginine paradox". *J Biol Chem* 272: 31213–31216, 1997.
34. **Mills JW and Mandel LJ.** Cytoskeletal regulation of membrane transport events. *FASEB J* 8: 1161–1165, 1994.
35. **Mombouli J and Vanhoutte P.** Kinins and endothelial control of vascular smooth muscle. *Annu Rev Pharmacol Toxicol* 35: 679–705, 1995.
36. **Moncada S, Palmer R, and Higgs EA.** Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 43: 109–142, 1991.
37. **Nakatsubo N, Kojima H, Kikuchi K, Nagoshi H, Hirata Y, Maeda D, Imai Y, Irimura T, and Nagano T.** Direct evidence of nitric oxide production from bovine aortic endothelial cells using new fluorescence indicators: diaminofluoresceins. *FEBS Lett* 427: 263–266, 1998.
38. **Patel JM, Zhang J, and Block ER.** Nitric-oxide-induced inhibition of lung endothelial cell nitric oxide synthase via interaction with allosteric thiols: role of thioredoxin in regulation of catalytic activity. *Am J Respir Cell Mol Biol* 15: 410–419, 1996.
39. **Pieper GM.** Review of alterations in endothelial nitric oxide production in diabetes: protective role of arginine on endothelial dysfunction. *Hypertension* 31: 1047–1060, 1998.
40. **Posch K, Schmidt K, and Graier WF.** Selective stimulation of L-arginine uptake contributes to shear stress-induced formation of nitric oxide. *Life Sci* 64: 663–670, 1999.
41. **Prat AG, Xiao Y-F, Ausiello DA, and Cantiello HF.** cAMP-independent regulation of CFTR by actin cytoskeleton. *Am J Physiol Cell Physiol* 268: C1552–C1561, 1995.
42. **Sase K and Michel T.** Expression and regulation of endothelial nitric oxide synthase. *Trends Cardiovasc Med* 7: 28–37, 1997.
43. **Schmidt K, Werner-Felmayer G, Mayer B, and Werner ER.** Preferential inhibition of inducible nitric oxide synthase in intact cells by the 4-amino analogue of tetrahydrobiopterin. *Eur J Biochem* 259: 25–31, 1999.
44. **Schott CA, Gray GA, and Stoclet JC.** Dependence of endotoxin-induced vascular hyporeactivity on extracellular L-arginine. *Br J Pharmacol* 108: 38–43, 1993.
45. **Sessa WC, Garcia-Cardena G, Liu J, Keh A, Pollock JS, Bradley J, Thiru S, Braverman I, and Desai KM.** The Golgi association of endothelial nitric oxide synthase is necessary for the efficient synthesis of nitric oxide. *J Biol Chem* 270: 17641–17644, 1995.
46. **Shaul PW and Anderson RGW.** Role of plasmalemmal caveolae in signal transduction. *Am J Physiol Lung Cell Mol Physiol* 275: L843–L851, 1998.
47. **Shaul PW, Smart EJ, Robinson LJ, German Z, Yuhanna IS, Ying Y, Anderson RGW, and Michel T.** Acylation targets endothelial nitric oxide synthase to plasmalemmal caveolae. *J Biol Chem* 271: 6518–6523, 1996.
48. **Smart EJ, Ying Y, Mineo C, and Anderson RGW.** A detergent-free method for purifying caveolae membrane from tissue culture cells. *Proc Natl Acad Sci USA* 92: 10104–10108, 1995.
49. **Sobrevia L, Cesare P, Yudilevich DL, and Mann GE.** Diabetes-induced activation of system y^+ and nitric oxide synthase in human endothelial cells: association with membrane hyperpolarization. *J Physiol (Lond)* 489: 183–192, 1995.
50. **Spector I, Braet F, Shochet NR, and Bubb MR.** New anti-actin drugs in the study of the organization and function of the actin cytoskeleton. *Microsc Res Tech* 47: 18–37, 1999.
51. **Venema VJ, Marrero MB, and Venema RC.** Bradykinin-stimulated protein phosphorylation promotes endothelial nitric oxide synthase translocation to the cytoskeleton. *Biochem Biophys Res Commun* 226: 703–710, 1996.
52. **Wang Q, Patton WF, Chiang ET, Hechtman HB, and Shepro D.** Filamin translocation is an early endothelial cell inflammatory response to bradykinin: regulation by calcium, protein kinases and protein phosphatases. *J Cell Biochem* 62: 383–396, 1996.
53. **Wang Q, Patton WF, Hechtman HB, and Shepro D.** A novel anti-inflammatory peptide inhibits endothelial cell cytoskeletal rearrangement, nitric oxide synthase translocation, and paracellular permeability increases. *J Cell Physiol* 172: 171–182, 1997.
54. **Woodard MH, Dunn WA, Laine RO, Malandro M, McMahon R, Simell O, Block ER, and Kilberg MS.** Plasma membrane clustering of system y^+ (CAT-1) amino acid transporter as detected by immunohistochemistry. *Am J Physiol Endocrinol Metab* 266: E817–E824, 1994.
55. **Wu Z, Wong K, Glogauer M, Ellen RP, and McCulloch CA.** Regulation of stretch-regulated intracellular calcium transients by actin filaments. *Biochem Biophys Res Commun* 261: 419–425, 1999.
56. **Zharikov SI and Block ER.** Characterization of L-arginine uptake by plasma membrane vesicles isolated from cultured pulmonary artery endothelial cells. *Biochim Biophys Acta* 1369: 173–183, 1998.
57. **Zharikov SI and Block ER.** Association of L-arginine transporter with fodrin: implications for hypoxic inhibition of L-arginine uptake. *Am J Physiol Lung Cell Mol Physiol* 278: L111–L117, 2000.
58. **Zharikov SI, Herrera H, and Block ER.** Role of membrane potential in hypoxic inhibition of L-arginine uptake by lung endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 272: L78–L84, 1997.