Nitric Oxide Attenuates Signal Transduction
Possible Role in Dissociating Caveolin-1 Scaffold

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Abstract—Caveolae harbor different serpentine receptors, intracellular components of signaling cascades, and certain enzymes, including endothelial nitric oxide synthase (eNOS). The regulation of eNOS activity by Ca^{2+}/calmodulin and caveolin has been described. We have previously demonstrated that nitric oxide (NO) can modulate signaling initiated via receptors localized to caveolae. In the present study, we show that NO donors induced an increase in the monomeric form of this scaffolding protein in cultured endothelial cells, the effect mimicked by 8-bromo cGMP. Proximity imaging of endothelial cells transfected with the thermotolerant green fluorescent protein–caveolin-1 construct demonstrated that sodium nitroprusside resulted in the increased fluorescence ratio of 410:470 nm, consistent with the distancing of fluorescently tagged caveolin-1. Pulse labeling of endothelial cells with cholera toxin B subunit indicated that sodium nitroprusside reversibly decreased its binding. Signaling via G protein–coupled receptors resident to caveolae was inhibited by pretreatment with NO donor. The data demonstrate that NO modulation of cell signaling is accomplished in part by regulating the state of caveolin-1 oligomerization. NO-induced attenuation of signaling involves reversible dissociation of caveolin scaffold, thus providing both spatial and temporal modulation of signal transduction. (Circ Res. 2001;88:229-236.)

Key Words: caveolae ■ green fluorescent protein ■ cytosolic calcium concentration ■ nitric oxide

Caveolae have been proposed to play an important role in cell signaling. Endothelin-A and bradykinin B2 receptors, epidermal growth factor (EGF) and platelet-derived growth factor receptors, mitogen-activated protein kinase, src-family nonreceptor tyrosine kinases, G proteins, protein kinase C, and cationic amino acid transporter-1 (CAT-1) arginine transporter, among others, are concentrated in these signalosomes.1-7 Caveolar-coat protein caveolin8 forms high-molecular-weight Triton-insoluble complexes through oligomerization mediated by N-terminal residues 61-101.9,10 Endothelial nitric oxide synthase (eNOS) is anchored in part to caveolae by cotranslational N-myristoylation and posttranslational palmitoylation11-15 and catalyzes the NADPH-dependent production of nitric oxide (NO) in the process of converting L-arginine to L-citrulline. Caveolin-1 coimmunoprecipitates with eNOS; their interaction occurs via the caveolin-1 scaffolding domain (N-terminal residues 81-101) and seems to result in the inhibition of the enzymatic activity.12,16 The inhibitory conformation of eNOS is reversed by the addition of excess Ca^{2+}/calmodulin.17-19 Agonists stimulating eNOS, eg, bradykinin, have been shown to promote depalmitoylation and subsequent subplasmalemmal translocation of the enzyme.7,20

We and others have previously demonstrated that NO can modulate signaling initiated via receptors, which have recently been localized to caveolae.4,21 In view of the above spatial relationship between the oligomeric caveolin-1 and eNOS activity, we inquired whether eNOS activity and generation of NO could modulate signal transduction cascades harbored in caveolae and whether this effect could be mediated through changes in the spatial or temporal modulation of signaling, eg, by affecting the state of oligomerization of caveolar scaffolding protein, caveolin-1. This possibility was addressed in the present study.

Materials and Methods

Cell Cultures
Human umbilical vein endothelial cells (HUVECs), used between passages 3 and 8, and SV-40–immortalized rat renal microvascular endothelial cells (RMVECs)22 were cultured in EBM-2–defined medium containing 2% FBS (Clonetics); rat renal microvascular smooth muscle cells were maintained in RPMI 1640 medium supplemented with 5% FCS.

Western Blot Analysis
Cells lysates were prepared using ice-cold lysis buffer containing 20 mmol/L Tris, pH 7.8, 140 mmol/L NaCl, 1 mmol/L EDTA, complete
miniprotease inhibitor cocktail (Boehringer Mannheim), 60 mmol/L octyl glucoside, and 1 mmol/L orthovanadate. The protein concentration of the lysates was determined with Pierce BCA protein assay against BSA standards. Boiling of samples was omitted, unless stated otherwise, to preserve the actual state of caveolin-1 oligomerization. Total protein of 20 μg from each sample was run on 4% to 20% Tris-glycine gel (Novex), transferred to Immobilon-P (Millipore Corp), and blocked with 1% casein in PBS. Results were quantified by scanning films and determining band density using Scion-image β-3b software.

**Velocity Sedimentation**

HUVECs were washed with ice-cold PBS and scraped into the lysis buffer of the specified composition: 20 mmol/L Tris, pH 8, 150 mmol/L NaCl, 1% Triton X-100, 60 mmol/L octyl glucoside, and protease inhibitors. After centrifugation at 22,000 rpm for 10 minutes at 4°C, the supernatants were loaded on the top of a linear 5% to 30% sucrose gradient and ultracentrifuged at 58,000 rpm for 16 hours in a Beckman swinging bucket SW-65 rotor. Thirteen fractions were collected from the top gradient. Proteins, precipitated with TCA, were resuspended in SDS-PAGE sample buffer, boiled for 5 minutes, separated by gel electrophoresis, and immunoblotted with polyclonal anti–caveolin-1 antibody. Fraction of Triton X-100-insoluble membranes were incubated on ice for 30 minutes, followed by dounce homogenizer. The lysates were mixed with an equal volume of 2.5 mol/L sucrose. The samples were overlaid with a 10% to 30% linear sucrose gradient and centrifuged for 21 hours at 29,000 rpm in a Beckmann SW 65 rotor. Fractions were collected from the top, and the total protein in each fraction was precipitated with TCA. Precipitates were dissolved in SDS-PAGE sample buffer, boiled for 5 minutes, separated in 4% to 20% Tris Glycine gels, and immunoblotted with polyclonal anti–caveolin-1 antibody and anti-GFP antibody.

**Binding of Cholera Toxin B by Intact and Sodium Nitroprusside–Treated Cells**

HUVECs or RMVECs grown on glass coverslips were incubated for the specified periods of time with 100 μmol/L sodium nitroprusside (SNP). After washing with ice-cold PBS, cells were incubated at 4°C for 20 minutes with FITC-labeled cholera toxin B (CTxB) (20 μg/mL in PBS with 1% BSA). After washing with PBS, cells were fixed with 4% paraformaldehyde, washed with PBS and ddH2O, mounted, and viewed using fluorescence microscopy.

**Construction of Caveolin–Enhanced Green Fluorescent Protein (EGFP) and Caveolin-Thermotolerant–GFP Expression Vectors**

The full open-reading frame of the human caveolin-1 (nucleotides 35 to 571) was cloned from HUVEC Ag11phage cDNA library by polymerase chain reaction (Clonetech) using appropriate primers containing XhoI and BamHI restriction sites at 5′ and 3′ with stop-codon mutated. cDNA was digested with XhoI and BamHI and ligated in sense orientation at the appropriate cloning site of the pEGFP-N1 plasmid using rapid DNA ligation kit (Boehringer Mannheim). Ligated plasmids were used to transform One Shot INVαF cells (Invitrogen). Transformed cells were selected for kanamycin resistance, propagated, and isolated. The construct was sequenced, and the authenticity of the product was confirmed.

PGEX plasmid containing thermotolerant GFP (ttGFP) was used to generate caveolin-ttGFP constructs. ttGFP cDNA was digested with NotI and BamHI and cloned into the EGFP vector with EGFP removed by NotI and BamHI. The construct was sequenced, and the authenticity was confirmed. The polybasic domain and isoprenylation sequence of p21 K-ras(β), sufficient for targeting and the authenticity was confirmed. The polybasic domain and isoprenylation sequence of p21 K-ras(β), sufficient for targeting and the authenticity was confirmed. The polybasic domain and isoprenylation sequence of p21 K-ras(β), sufficient for targeting and the authenticity was confirmed. The polybasic domain and isoprenylation sequence of p21 K-ras(β), sufficient for targeting and the authenticity was confirmed.

**Fraction of Triton X-100–Insoluble Membranes**

Miniprotease inhibitor cocktail (Boehringer Mannheim), 60 mmol/L octyl glucoside, and protease inhibitors, HUVECs were lifted with a rubber policeman in 1% Triton X-100–containing buffer. The cells were incubated on ice for 30 minutes, followed by homogenization with dounce homogenizer. The lysates were mixed with an equal volume of 2.5 mol/L sucrose. The samples were overlaid with a 10% to 30% linear sucrose gradient and centrifuged for 21 hours at 29,000 rpm in a Beckmann SW 65 rotor. Fractions were collected from the top, and the total protein in each fraction was precipitated with TCA. Precipitates were dissolved in SDS-PAGE sample buffer, boiled for 5 minutes, separated in 4% to 20% Tris Glycine gels, and immunoblotted with polyclonal anti–caveolin-1 antibody and anti-GFP antibody.

**Proximity Imaging**

Intravital fluorescence microscopy of HUVECs or RMVECs was performed using a Nikon epifluorescence inverted microscope (Diaphot) equipped with a SIT camera (Hamamatsu) and enclosed in a temperature-controlled incubator (Nikon). Cells were illuminated at alternating wavelengths of 410 and 470 nm, with intervals ranging from 1 to 5 minutes, using an automatic shutter (Lambda 10-2, Sutter Instruments) interfaced to Image-1 software (Universal Imaging). Images were collected at the wavelength of 530 nm using an appropriate dichroic mirror, stored, and analyzed using an Image-1 software. Confocal microscopy was performed using an Odyssey system (Noran Instruments) equipped with Metamorph software (Universal Imaging) and analyzed using a Silicon Graphic system.

**Cytosolic Calcium Concentration**

HUVECs or smooth muscle cells (SMCs) were grown on glass coverslips, transfected with GFP–caveolin-1 construct, when necessary, and loaded with 2 μmol/L fura-2-AM. Changes in [Ca2+], were examined with a spectrofluorometer (Photon Technology International) at alternating excitation wavelengths 345 and 380 nm and emission wavelength 510 nm, with derivation of 345:380 ratio, as previously detailed.

**NO-Selective Microelectrode Measurements**

The NO concentration in cells bathed in Krebs-Ringer-HEPES was monitored with porphyrin-electroplated, Nafton-coated, carbon fiber electrodes (30 μm outer diameter), which were manufactured according to Bio-Logic Instruments’ instructions. Measurements were made using constant potential amperometry (0.7 mV) using a highly sensitive potentiostat (InterMedical). The resulting signal was low-pass-filtered at 0.5 Hz and sampled every 2 seconds. A microelectrode mounted on a micromanipulator was positioned 5 to 10 μm away from cultured cells under visual control on an inverted microscope. At the completion of experiments, electrodes were calibrated using different dilutions of NO-saturated PBS.

**Results**

Exposure of the endothelial cells to SNP, an NO donor, resulted in an increase in the 22-kDa monomeric form of caveolin-1 (Figures 1A and 1B). Treatment of HUVECs with 0.5 to 100 μmol/L SNP resulted in an increase in the 22-kDa monomeric form of caveolin-1 as early as 2 minutes and peaked at 10 to 30 minutes, returning to near baseline by 90 minutes (Figure 1C). Effects of SNP were mimicked by a cell-permeable 8-bromo cGMP (Figure 1D). Endogenous NO production by A23187-activated eNOS resulted in a similar increase in the monomeric caveolin-1, which was partially inhibited by the pretreatment with NOS inhibitor Nω-nitro-L-arginine methyl ester (Figure 2). The above observations suggest that application of NO donors or activation of endogenous NO production via activation of guanylate cyclase leads to the increase in the fraction of monomeric caveolin-1.

These findings were additionally confirmed using velocity sedimentation analysis. As shown in Figure 3, immunodetectable caveolin-1 was confined to high-molecular-weight frac-
tions in resting cells but has become detectable in the low-molecular-weight fractions as soon as 10 minutes after application of SNP or S-nitroso-N-acetyl-penicillamine (SNAP) and returned to background 90 minutes later. During these transitions, immunodetectable eNOS was confined to the high-molecular-weight fractions corresponding to caveolin-1 scaffold.

One of the recently described fluorescence techniques that allows assessment of the tightness of interaction between homooligomeric proteins, termed proximity imaging (PRIM), was used next to obtain independent data on the ability of NO to interfere with oligomeric structure of caveolin. PRIM relies on the fact that ttGFP can undergo shifts in its excitation ratio when 2 copies of the protein are brought into close proximity, such as via fusion to proteins that self-associate. Whereas monomeric proteins always display a constant excitation ratio, homooligomerization results in either an increase or decrease in the excitation ratio of ttGFP.

Quality-control studies assessing the validity of caveolin-GFP construct are presented in Figure 4. Transfection of HUVECs with this vector resulted in a punctate fluorescence pattern, which colocalized with the anti–caveolin-1 immunostaining. Moreover, caveolin-GFP was immunodetected in the Triton X–insoluble fraction, obtained from transiently transfected RMVECs (Figure 4B). Antibodies against GFP revealed a single band with the apparent molecular mass of ~50 kDa, whereas blotting with anti–caveolin-1 antibodies revealed 2 bands, the endogenous caveolin-1 and caveolin-GFP, both colocalizing to the same light membrane fractions. These data indicate that the GFP–caveolin-1 construct used in this study was appropriately expressed by endothelial cells. The fact that it was recoverable from the Triton X–insoluble fraction argues that it was capable of oligomerization.

To determine whether NO-induced dissociation of caveolin complexes could be monitored intravitaly in RMVECs or HUVECs, we fused the protein to ttGFP and performed PRIM. After transfection, caveolin-ttGFP was localized in a punctate pattern throughout the cytoplasm as well as at the plasma membrane. These cells displayed a 0.43±0.04, which increased to 0.87±0.14 by 10 minutes after application of 100 μmol/L SNP (n=8; P<0.05). To rule out the possibility that this shift in excitation ratio might reflect a direct effect of SNP or NO per se, irrespective of the caveolin-1 moiety, we performed the same experiments in cells expressing ttGFP alone. The data demonstrated that the excitation ratio remained stable at 0.6±0.02 throughout the experiments. Furthermore, additional control experiments in cells transfected with ttGFP targeted to the plasma membrane via palmitoylation or isoprenylation consensus sequences (Figures 5C and 5D) also did not undergo SNP-dependent excitation ratio shifts: the ratios remained stable at 0.47±0.04 and 0.3±0.03, respectively. The reason for the difference in the excitation ratios of palmitoylated and isoprenylated ttGFP is presently unclear, but it may reflect differences in the degree of self-association provided by each lipid modification. In either case, however, incubation with SNP did not affect their excitation ratio. The excitation ratio shift undergone by ttGFP–caveolin-1 on SNP treatment thus reflects transient changes in intermolecular distance or angle separating caveolin molecules, consistent with transient dissociation and reassociation of caveolin-1 oligomers after exposure to the NO donor.
The spatial relationship between caveolin-1, various serpentine receptors, and eNOS has been previously established.4,11–13, The emerging paradigm is that some receptors, their respective intracellular signal transducers, and eNOS are enriched on the cytoplasmic caveolar surface by virtue of palmitoylation- or myristylation-induced anchorage to caveolin-1 (but not caveolin-2).2,3,6,11,15 The controversy exists, however, regarding the functional consequences of such an organization of signaling molecules. There is evidence that binding of G proteins or eNOS to this protein of caveolar coat is associated with their inactivation.14,19 On the other hand, the spatial proximity of the elements integral to signaling cascades seems to facilitate their interaction when a stimulus arrives. If the application of NO donors does indeed dissociate oligomeric caveolin-1, as suggested by the data shown above, it is conceivable that NO pretreatment could disrupt signaling through receptors resident to caveolae, possibly by distancing elements of signaling cascades. One such receptor is represented by the B2 bradykinin receptor.7 Changes in cytosolic calcium concentration ([Ca^{2+}]_{i}) were monitored as a downstream read-out system to test this hypothesis. When fura-2–loaded HUVECs were stimulated with SNP and challenged with 10 μmol/L bradykinin, [Ca^{2+}]_{i} transients were almost completely abrogated (the amplitude of responses was diminished by 72±14%; P, 0.05), in striking contrast to the typical responses recorded from nonpretreated cells (Figure 6A). Notably, [Ca^{2+}]_{i} responses elicited by ionomycin were preserved after SNP pretreatment.

As mentioned above, the endothelin A receptor is localized to caveolae in vascular smooth muscle cells,13 where it is coupled to the activation of phospholipase C and elevation of [Ca^{2+}]_{i}.4,21,31 To test the possibility that NO interferes with endothelin-1 (ET-1) signaling in SMCs as well, fura-2–loaded SMCs were stimulated with 10 nmol/L ET-1 after pretreatment with SNP. As shown in Figure 6B, the typical ET-1–induced [Ca^{2+}]_{i} transients observed in control cells were inhibited in SMCs pretreated with the NO donor by 81±15% (P<0.05). This is in accord with our earlier detailed analysis of the sites of NO regulation of ET-1–induced [Ca^{2+}]_{i} transients in CHO cells transfected with ET_{A} receptor.21

Hence, the data presented here support the idea that the scaffolding function of caveolin-1 modulates signaling via receptors resident to caveolae. Because the above data document the dissociation of caveolin-1 oligomers after application of SNP and this NO donor interferes with the signaling through such receptor complexes as endothelin A and bradykinin B_{2}, these findings are consistent with the NO-induced...
Recent observations by Orlandi and Fishman on the effects of filipin in CaCo-1 cells have also demonstrated that this sterol-binding agent disrupted the cholera toxin–induced cAMP accumulation. Ganglioside GM1 is a receptor for CTxB subunit, largely clustered in caveolae, and has been demonstrated in various cells, including the vascular endothelium. Binding of CTxB to this receptor represents a convenient tool to study the integrity of caveolae-associated signaling. In the next series of experiments, cultured endothelial cells were incubated for 20 minutes with 20 μg/mL of CTxB subunit conjugated to FITC at 4°C, and, after removal of the unbound ligand by washing and fixation with paraformaldehyde, cell-associated fluorescence intensity was examined using quantitative image analysis of pulse-labeled cells. As shown in Figure 7, when pulse-labeling was performed 10 minutes after application of 100 μmol/L SNP, the intensity of fluorescence associated with HUVECs was significantly decreased. Fluorescence intensity of HUVECs pulse-labeled with CTx-FITC has recovered 60 minutes after application of SNP.

The phenomenon of L-arginine–induced NO production (L-arginine paradox) has been suggested to be related to the proximity of the CAT-1 amino acid transporter to eNOS in caveolae. Therefore, it is conceivable that dissociation of caveolin-1 may interfere with L-arginine–induced generation of NO, especially under experimental conditions when the substrate is not present in the incubation medium. RMVECs bathed in Krebs-HEPES buffer, without added L-arginine, were repeatedly stimulated by cycles of addition/washout of 100 μmol/L L-arginine, and NO generation was monitored using an NO-selective microelectrode. The first application of L-arginine stimulated NO production by RMVECs (Figure 8A). After changing the bathing solution, L-arginine was reapplied. The amplitude of NO responses was diminished when the interval between stimuli was 5 and 10 minutes (despite the fact that A23187 elicited unperturbed responses), but at intervals of 30 and 60 minutes, NO responses recovered the initial amplitude. Moreover, when endothelial cells were pretreated with 8-bromo cGMP, L-arginine–induced generation of NO was reduced from baseline of 70 ± 6 to 39 ± 6 nmol/L (n = 4, P < 0.05). Furthermore, pretreatment with an inhibitor of guanylyl cyclase, 1H-[1,2,4]oxadiazole[4,3-
Aquinoxalin-1-one (ODQ), 10 μmol/L for 20 minutes, restored almost completely the amplitude of NO responses to repeated L-arginine administration after an interval of 5 minutes (Figure 8B). The observed transient responses to L-arginine in vivo are in sharp contrast to in vitro NO generation by the recombinant eNOS. As shown in Figure 8C, addition of L-arginine to the eNOS, incubated in a buffer containing 100 nmol/L calmodulin, 500 μmol/L NADPH, 5 μmol/L FAD, 5 μmol/L FMN, 1 μmol/L tetrahydrobiopterin, and 10 μmol/L calcium chloride, as previously reported, resulted in a protracted NO generation by the enzyme and showed little downregulation of its activity. These findings additionally buttress the proposed in vivo effect of NO, mediated via cGMP, on the state of caveolin-1 oligomerization and indicate that the efficient termination of eNOS signaling requires additional mechanisms dependent on cellular processing of the enzyme.

Discussion

The data presented here demonstrate in primary cultures and immortalized endothelial cells that NO results in a reversible and cGMP-dependent increase in the fraction of monomeric caveolin-1. Three lines of experimental evidence support this conclusion: first, Western blot analysis of endothelial cells subjected to the exogenous or endogenous NO; second, shift of caveolin-1 to the lighter fractions on the sucrose equilibrium density centrifugation; and third, PRIM analysis demonstrating the increased 410:470 ratio of caveolin-ttGFP fluorescence in transiently transfected endothelial cells. Collectively, these lines of experimental evidence strongly suggest that NO interferes with the integrity of caveolin-1 scaffolding function. Three independent sets of functional tests designed to challenge this effect of NO were performed. These included the following: (1) calcium signaling, as a downstream read-out event characterizing signal transduction through G protein–coupled membrane receptors known to be harbored in caveolae; (2) binding of CTXb subunit to its ganglioside receptor harbored in caveolae of endothelial cells; and (3) effects of L-arginine on NO production by cultured endothelial cells. Findings of these independent series of experiments are consistent with the idea that NO results in a reversible dissociation of the downstream signal-
ing through the resident caveolar receptors, as well as inhibition of 1-arginine paradox and binding of CTxB subunit. Although the interpretation of each of these tests by itself is quite complex, collectively, these data suggest that the integrity of the scaffolding function of caveolin-1 is potentially important for the spatial organization of signaling systems localized to caveolae and that NO modulates signal transduction by dissociating caveolin-1 oligomers. Recent studies in animals with pacing-induced heart failure have shown the increased density of caveolae and increased expression of caveolin-3 and eNOS in myocardocytes, associated with augmented NO signaling, thus paving the way to additional investigation of complex relationships between this scaffolding protein and the enzyme.36

The precise mechanism whereby NO interferes with the oligomeric state of caveolin-1 is not clear. Several possible scenarios can be envisioned. NO effects on caveolin-1 and its scaffolding might involve changes in the state of caveolin-1 phosphorylation, because NO has been shown to inhibit the activity of phosphatases.37,38 It is also possible that NO affects dynamin and internalization of caveolae,39,40 or it may interfere with caveolar budding by other yet unknown mechanisms. Our preliminary electron microscopic findings of the decline in the number of plasma membrane-associated caveolae and increased number of vesicles and vacuoles (not shown) are consistent with this mode of NO action. Such an action would be perfectly fitting the present view of caveolae as dynamic structures being constantly recycled between the plasma membrane, endosomes, and the trans-Golgi network.41,42,43 If this is the case, caveola-harbored receptors, elements of signaling pathways, and eNOS may undergo parallel internalization and recycling, tracking the caveolin-1 pathway. Indeed, eNOS has been previously localized to the cytoplasmic vesicles and trans-Golgi network,44,45 suggesting that the above recycling path does exist.

The existing model of functional shuttling of eNOS between the caveolin-associated state with the suppressed NO generation and the calmodulin-activated state characterized by the augmented activity of the enzyme46 may gain some mechanistic details on the basis of data presented herein. Specifically, the model postulates that the dissociation of calmodulin-eNOS complex follows the recovery of cytosolic calcium concentration and precedes eNOS reassociation with caveolin-1. Our data suggest that the second stimulus, namely NO produced by the stimulated enzyme, actively participates in the negative feedback regulation by dissociating the scaffolding oligomeric structure of caveolin-1 and distancing the elements of signaling cascades harbored in caveolae. It is conceivable that the same mechanism shuts down NO production by distancing eNOS from CAT-1.

In conclusion, the topological proximity of [Ca\(^{2+}\)]\(_i\)-mobilizing receptors, G proteins, and eNOS within the caveolae has, most probably, a dual function. On the one hand, it may facilitate signal transduction by virtue of compartmentalization of the elements of signaling cascade. On the other hand, stimulation of NO production may disintegrate this topological proximity, resulting in the termination of signal (autocrine regulation of signal cascade). In addition, NO acting in a paracrine manner may also attenuate signaling through caveola-harbored receptors on vascular smooth muscle cells. Both phenomena underscore the possibility of a novel type of regulation of signal transduction, a topological regulation, and the ability of NO to modulate signaling both spatially and temporally.

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References


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