Direct Interaction between Endothelial Nitric-oxide Synthase and Dynamin-2

IMPLICATIONS FOR NITRIC-OXIDE SYNTHASE FUNCTION*

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Endothelial nitric-oxide synthase (eNOS) is regulated in part through specific protein interactions. Dynamin-2 is a large GTPase residing within similar membrane compartments as eNOS. Here we show that dynamin-2 binds directly with eNOS thereby augmenting eNOS activity. Double label confocal immunofluorescence demonstrates colocalization of eNOS and dynamin in both Clone 9 cells cotransfected with green fluorescent protein-dynamin and eNOS, as well as in bovine aortic endothelial cells (BAEC) expressing both proteins endogenously, predominantly in a Golgi membrane distribution. Immunoprecipitation of eNOS from BAEC lysate coprecipitates dynamin and, conversely, immunoprecipitation of dynamin coprecipitates eNOS. Additionally, the calcium ionophore, A23187, a reagent that promotes nitric oxide release, enhances coprecipitation of dynamin with eNOS in BAEC, suggesting the interaction between the proteins can be regulated by intracellular signals. In vitro studies demonstrate that glutathione S-transferase (GST)-dynamin-2 quantitatively precipitates both purified recombinant eNOS protein as well as in vitro transcribed 35S-labeled eNOS from solution indicating a direct interaction between the proteins in vitro. Scatchard analysis of binding studies demonstrates an equilibrium dissociation constant ($K_d$) of 27.6 nM. Incubation of purified recombinant eNOS protein with GST-dynamin-2 significantly increases eNOS activity as does overexpression of dynamin-2 in ECV 304 cells stably transfected with eNOS-green fluorescent protein. These studies demonstrate a direct protein-protein interaction between eNOS and dynamin-2, thereby identifying a new NOS-associated protein and providing a novel function for dynamin. These events may have relevance for eNOS regulation and trafficking within vascular endothelium.

Endothelial nitric-oxide synthase (eNOS)$^1$ is a membrane-associated protein that is localized in the Golgi apparatus as well as within cholesterol-rich plasmalemmal vesicles, termed caveolae (1–3). The ability of eNOS to generate nitric oxide (NO) has traditionally been ascribed to agonist-stimulated calcium-dependent activation. However, during recent years several groups have demonstrated that activation of eNOS, both in conjunction with and independent of intracellular calcium flux, occurs through the allosteric binding of eNOS with neighboring regulatory proteins (4–6). In this regard, several eNOS-associated proteins have been identified, including the caveolae coat protein, caveolin, calmodulin, Hsp 90, and the bradykinin-2 receptor (6–9).

Dynamin-2 is a large ubiquitously expressed GTP-binding protein that targets to Golgi membranes and colocalizes with caveolin within caveolae (10). Although the function of dynamin is best characterized in membrane scission events (11–14), members of this family of proteins also modulate signaling pathways by means of distinct protein interactions (10, 15–17). In support of this concept, dynamin-1 binds and regulates the calcium-sensitive phosphatase, calcinexin, and the dynamin family of proteins interact with the SH3 domains of a variety of signaling proteins by virtue of a proline-rich domain (16–20).

Based on previously published studies indicating that both eNOS and dynamin-2 colocalize with caveolin and reside within similar membranes compartments (1, 2, 12–14), we examined whether these two proteins might interact in a functional manner. Here we demonstrate that pools of eNOS and dynamin bind within cells in a manner regulated by ionic stringency and intracellular signals and that in vitro the proteins bind directly in a stoichiometric and high affinity manner. Additionally, the interaction of dynamin with eNOS potentiates eNOS catalysis in cells and in vitro. These studies identify a new NOS-associated protein and provide a novel function for dynamin-2.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—All tissue culture and transfection reagents were obtained from Life Technologies, Inc. eNOS mAb and pAb were obtained from Transduction Laboratories (Lexington, KY). Golgi 58-kDa protein was obtained from Sigma. The dynamin-2 pAb (dyn2T) has been previously described (12). This antibody recognizes the proline-rich COOH-terminal tail unique to dynamin-2 and specifically precipitates this dynamin isoform from brain lysates. Calcium ionophore, A23187, was obtained from Sigma. 1H-Labeled L-arginine and 35S-labeled methionine were obtained from Amersham Pharmacia Biotech.

cells: GST, glutathione S-transferase; NO, nitric oxide; L-NAME, L-nitroarginine methyl ester; mAb, monoclonal antibody; pAb, polyclonal antibody; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; IP, immunoprecipitation.

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† The abbreviations used are: eNOS, endothelial nitric-oxide synthase; GFP, green fluorescent protein; BAEC, bovine aortic endothelial.
**Expression and Purification of Recombinant eNOS and GST-Dynamin Fusion Protein—** Recombinant eNOS protein was purified from *Escherichia coli* as described previously (23, 24). In brief, bovine eNOS in the plasmid, pCW was coexpressed with pGroELS plasmid into *Escherichia coli* by incubating cells with eNOS pAb and Golgi 58-kDa protein mAb, and lysed by sonication with lysozyme 200 μg/ml in a buffer containing 20 mM Hpes (pH 7.2), 100 mM KCl, 2 mM MgCl, 1 mM dithiothreitol, 2 μM leupeptin, 1 mM PMSF, and 0.1% SDS, 0.1% Triton X-100. Specificity and quality of GST-dynamin was assessed by Coomassie staining of SDS-PAGE gels and Western blotting of transferred proteins as depicted in Fig. 4A.

**In Vitro Translation of [35S]Methionine eNOS—** [35S]Methionine eNOS was translated in rabbit reticulocyte lysates using the TNT Capped Reticulocyte Lysate System (Promega, Madison, WI). The reaction mix contained [35S]methionine (200 μCi/ml), [35S]methylamino (6.7 μCi/ml), or alternatively containing empty vector), SP6 RNA polymerase promoter, and [35S]methionine, was incubated at 30 °C for 90 min. Translation products were examined by SDS-PAGE analysis and autoradiography of dried gels. Binding assays using [35S]methionine eNOS were performed as described below.

**Confocal Immunofluorescence Microscopy—** BAE and Clone 9 cells (48 h after cotransfection with eNOS and GFP-dynamin) were fixed in 2% paraformaldehyde. Double-labeling immunofluorescence was performed in BAE as previously described in liver endothelial cells (25), by incubating cells with eNOS pAb and Golgi 58-kDa protein mAb, dynamin pAb and Golgi 58-kDa protein mAb, or alternatively eNOS mAb and dynamin mAb. Immunofluorescence in Clone 9 cells transfected with GFP-dynamin was performed by incubating cells in eNOS mAbs only. Primary antibodies were detected using fluorescein isothiocyanate- and Texas Red-coupled secondary antibodies. Washes were performed with phosphate-buffered saline, 0.1% bovine serum albumin after both primary and secondary antibody incubation. Cells were mounted in Anti-fade (Molecular Probes, Eugene, OR) and visualized using a confocal microscope (LSM 510, Zeiss, Germany).

**Immunoprecipitation and Western Blotting—** BAE were homogenized in a lysis buffer (50 mM Tris-HCl, 0.1 mM EGTA, 0.1 mM EDTA, 2 μM leupeptin, 1 mM PMSF, 1% (v/v) Nonidet P-40, 0.1% SDS, 0.1% deoxycholate (pH 7.5)). In some experiments, BAE were incubated in media containing 10 μM 22,187 or equal volume of vehicle (MeSO) for 10 min prior to lysis. Protein quantification of samples was performed using the Bio-Rad protein assay. eNOS immunoprecipitation was performed by 1-ml aliquots of protein lysate with excess eNOS mAb overnight (1:200 dilution) or alternatively with equal concentration of mouse IgG after preclearing of samples with Pansorbin as previously described (26). Alternatively, dynamin immunoprecipitation was performed by incubating lysates with excess dynamin pAb (5 μg/ml) overnight. Immunocomplexes were bound by incubating protein samples with Protein A beads for 1 h at 4 °C. Triplicate samples of bound proteins were extensively washed in a buffer (50 mM Tris-HCl, 0.1 mM EGTA, 0.1 mM EDTA, 2 μM leupeptin, 1 mM PMSF) containing 0 mM NaCl, 100 mM NaCl, or alternatively 1 M NaCl. Bound proteins were eluted by boiling samples in Laemmli loading buffer. Gel electrophoresis of proteins and Western blotting were performed as previously described (25), using eNOS pAb and dynamin pAb. Membranes were stained with Ponceau S or gels with Coomassie Blue to confirm equal protein loading. Densitometric analysis of autoradiographs was performed using Scion Image from the National Institutes of Health.

**In Vitro Binding Assays—** Increasing concentrations of recombinant eNOS or GST-dynamin beads (60–600 nM) were incubated overnight at 4 °C with GST-dynamin beads or, alternatively, GST beads alone in immunoprecipitation buffer in the absence of detergents. Bound proteins were washed in a buffer containing 50 mM Tris (pH 7.7), 200 mM NaCl, and 1 mM EDTA (8). Bound proteins were eluted with Laemmli buffer and used for gel electrophoresis. In vitro binding of GST-dynamin with full-length GST-dynamin was examined by incubating GST-dynamin beads (60–600 nM) with GST beads alone, with a fixed concentration of in vitro translated eNOS (3 μl of rabbit reticulocyte lysate) or, alternatively, by incubating increasing concentrations of in vitro translated eNOS (1–12 μl of rabbit reticulocyte lysate) with a fixed concentration of GST-dynamin beads, in 300 μl of a buffer containing 50 mM Tris-HCl, 0.1 mM EDTA overnight. Bound proteins were washed three times with a buffer containing 50 mM Tris-HCl, 1 mM EDTA and 0.5 M NaCl. GST beads were used for gel electrophoresis. Binding studies with GST-dynamin and recombinant eNOS were analyzed by SDS-PAGE and Western blot analysis, whereas binding studies with GST-dynamin and 35S-eNOS were examined by SDS-PAGE and autoradiography of dried gels. Quantification of autoradiographs was performed by densitometric analysis using Scion Image. Estimation of equilibrium dissociation constant (Kd) was performed by incubating a fixed concentration of GST-dynamin beads (5 nM) with purified recombinant eNOS (0–320 nM) premixed with proportionate volume of 35S-eNOS (0–16 μl) used as a radiolabel tracer (the protein concentration of the radiolabel tracer is negligible compared with the protein concentration of the recombinant protein). Bound radioactive counts were measured directly by scintillation counting. For determination of bound and free recombinant eNOS, Kd was calculated by Scatchard plot analysis of bound and free levels of recombinant eNOS.

**NOS Activity Assays—** NOS activity from recombinant eNOS protein and eNOS-GFP ECV 304 cell lysates was assessed by measuring the conversion of [H]labeled l-arginine to [H]labeled l-citrulline in the presence and absence of l-nitroarginine methyl ester (l-NAM). As described previously, freshly isolated cells were incubated with eNOS pAb and Golgi 58-kDa protein mAb. NOS activity was determined by incubating recombinant proteins or duplicate samples of cell lysate were incubated with a buffer containing 1 mM NADPH, 3 μM tetrahydrobiopterin, 100 mM calmodulin, 2.5 mM CaCl2, 10 μM l-arginine and l-[3H]arginine (0.2 μCi) at 37 °C for 20 min in the presence and absence of 1 mM l-NAM. The reaction mix was terminated by the addition of 1 ml of cold stop buffer (20 mM Hepes, 2 mM EDTA, 2 mM EGTA (pH 5.5)) and passed over a Dowex AG 50WX-8 resin column. Radioabeled counts per min of generated l-citrulline were measured and used to determine l-NAM inhibitable NOS activity.

**Statistical Analysis—** All data are given as mean ± S.E. Data were analyzed using paired and unpaired Student’s t tests and one-way analysis of variance.

**RESULTS**

eNOS Colocalizes with Dynamin within Cells—Both eNOS and dynamin appear to reside within similar subcellular compartments based on previous studies examining subcellular localization of each of the proteins (12, 12–14). To determine whether eNOS and dynamin colocalized within cells, we performed confocal immunofluorescence microscopy in Clone 9 cells heterologously expressing eNOS and dynamin as well as in BAE which express both proteins endogenously. As seen in Fig. 1A, in Clone 9 cells transiently transfected with eNOS and GFP-dynamin, NOS protein is detected predominantly in a
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perinuclear distribution. GFP-dynamin is also detected in a perinuclear distribution with additional fluorescent signal detected within distinct punctate areas within the cytoplasm and plasma membrane (Fig. 1B). As seen in the merged image (Fig. 1C), there is colocalization of the two proteins in a perinuclear distribution. To examine the subcellular distribution and colocalization of endogenously expressed eNOS and dynamin, we next performed double labeling immunofluorescence microscopy in BAEC. In BAEC, eNOS is detected in a perinuclear pattern with some protein detected within distinct regions of plasma membrane (Fig. 1D). In Fig. 1E, immunolocalization of dynamin in BAEC demonstrates a similar distribution of protein as observed in Clone 9 cells, and as observed in the merged image in Fig. 1F, colocalization of the two proteins in BAEC is observed in predominantly a perinuclear pattern (small arrow) as well as in specific domains of plasma membrane (large arrow). Next, we further examined the nature of this prominent perinuclear area of colocalization in BAEC by examining the colocalization of eNOS and alternatively dynamin, with the Golgi marker, Golgi 58-kDa protein. eNOS and Golgi 58-kDa protein (Fig. 1, G and H, respectively) are detected in a perinuclear distribution and colocalization of proteins is detected, indicating that pools of eNOS protein reside within Golgi membranes as previously noted (Fig. 1I, small arrow) (1, 27). Note that distinct pools of eNOS in plasma membrane do not colocalize with Golgi 58-kDa protein, demonstrating the specificity of Golgi 58-kDa protein mAb (Fig. 1, large arrow). Next we examined localization of dynamin (Fig. 1J) and Golgi 58-kDa protein (Fig. 1K) in BAEC. As seen in Fig. 1L, pools of dynamin also colocalize with Golgi 58-kDa protein. No fluorescence was detected in negative control slides in which serum was substituted for the primary antibody or in cells incubated with secondary antibody alone (data not shown). These studies suggest that eNOS and dynamin colocalize predominantly within Golgi membranes of cells.

eNOS and Dynamin Bind in a Specific Manner within Cells—Based on the prominent colocalization of eNOS and dynamin particularly within BAEC, we next examined whether the two proteins interact biochemically. For this purpose, detergent-soluble lysates were prepared from BAEC, and eNOS protein was immunoprecipitated under nondenaturing conditions. As seen in Fig. 2A, immunoprecipitation of eNOS coimmunoprecipitates dynamin (lane labeled 0 mM NaCl). As seen in Fig. 2B, immunoprecipitation of dynamin conversely coimmunoprecipitates eNOS from BAEC extracts (lane labeled 0 mM NaCl). As observed in Fig. 2, A and B, coprecipitation of eNOS and dynamin is maintained in the presence of an increase in the ionic strength of the wash buffer to 100 mM NaCl (lane labeled 100 mM NaCl). However, when beads are washed in the presence of 1 mM NaCl, the interaction between the two proteins is markedly diminished suggesting an ionic strength protein-protein interaction (lane labeled 1 mM NaCl). We next examined the specificity of binding and pools of bound protein in cells, by substituting nonimmune sera for eNOS mAb during the immunoprecipitation and by comparing the pools of dynamin bound to eNOS with the total pool of dynamin in the lysate, respectively. As seen in Fig. 2C, ~5% of the dynamin pool contained in the post-immunoprecipitation lysate (lane labeled post IP that contains 20 μl from the 1 ml of lysate) is bound to eNOS (lane IP) as assessed by the similar dynamin Western blot signal intensity in these two lanes. (Similar results were obtained when the pre-IP lysate (not shown) was analyzed for dynamin in place of the post-IP lysate as the total pool of dynamin is not markedly reduced by eNOS immunoprecipitation.) Also, as seen in lane NIS, neither eNOS nor dynamin is detected when mouse IgG is substituted for eNOS mAb during the immunoprecipitation procedure. These studies indicate that fractions of eNOS and dynamin coexist in a complex within cells.

Ionophore Potentiates Binding of Dynamin with eNOS—To determine whether binding between eNOS and dynamin within cells is regulated by intracellular signals, we stimulated BAEC with the calcium ionophore, A23187 (10 μM), an agonist

![Fig. 1](image-url)
Dynamin and Endothelial Nitric-oxide Synthase

which promotes NO release (1). Control cells were treated with vehicle. Detergent-soluble lysates were prepared, and eNOS protein was quantitatively immunoprecipitated. As seen in lanes labeled IP, from the representative blot (Fig. 3A), eNOS immunoprecipitated from lysates of BAEC incubated with the ionophore commmunoprecipitated markedly greater levels of dynamin as compared with equal amounts of eNOS immunoprecipitated from cells treated with vehicle (compare the intensity of the dynamin Western blot signal in lanes labeled IP under vehicle and A23187). As seen in the lanes labeled Post IP, after immunoprecipitation, eNOS is barely detected within 20 μl of the remaining supernatant, whereas the majority of the cellular pool of dynamin remains unbound to eNOS. In Fig. 3B, when the series of experiments (n = 3) are examined as a group by densitometric analysis, a significant increase in dynamin bound to eNOS is detected after stimulation of cells with A23187 (* indicates p < 0.05).

eNOS and Dynamin Interact Directly in Vitro—Coprecipitation between eNOS and dynamin from cell lysates may occur directly or through an adapter protein. To determine whether eNOS and dynamin are able to bind directly, we next examined whether eNOS in solution binds to dynamin in the form of a GST fusion protein. Fig. 4A demonstrates the purity and specificity of the GST-dynamin fusion protein. In the left panel, enrichment of a single 26-kDa protein eluted from GST beads, and the enrichment of a 130-kDa protein eluted from GST-dynamin beads is observed by Coomassie staining of an SDS-PAGE gel. The right panel of Fig. 4A demonstrates the specific Western blot signal for dynamin that is detected from purified GST-dynamin beads but not GST beads alone. To determine whether eNOS and dynamin bind directly in vitro and to quantify the relative pools of bound protein, we incubated GST dynamin beads or alternatively GST beads alone with increasing concentrations of purified recombinant eNOS protein in solution (60–300 nM). As seen in the representative Western blot in Fig. 4B, left panel, specific binding of purified eNOS with GST dynamin is detected, whereas GST beads alone do not coprecipitate eNOS from solution. Additionally, at a 1:1 molar ratio of GST-dynamin to eNOS (lane labeled 150 nM eNOS in Fig. 4B), a majority of the pool of available eNOS protein is bound to dynamin (compare lane labeled 150 nM eNOS to lane input, which represents the available eNOS protein in the 150 nM sample). Further increase in the available concentration of eNOS results in a smaller increase in eNOS binding (compare lane labeled 300 nM eNOS to lane labeled 150 nM eNOS). These observations are quantified in Fig. 4B, right panel, in which the densitometric intensity of the Western blot analysis is depicted graphically. Note that at a 1:1 molar ratio of eNOS and GST-dynamin (bar labeled 150 nM), the majority of available eNOS protein (bar labeled input) binds with dynamin. To establish further the specificity of this direct interaction, we next determined whether GST dynamin binds [35S]methionine-labeled eNOS transcribed in vitro in reticulocyte lysates by incubating varying concentrations of GST-dynamin beads or GST beads alone, with a fixed volume of [35S]eNOS (3 μl). In the representative autoradiogram developed from a dried SDS-PAGE gel in Fig. 4C, left panel, note that whereas GST does not bind [35S]eNOS, GST-dynamin binds [35S]eNOS in a concentration-dependent manner. Again note that the majority of the available [35S]eNOS in solution is bound to dynamin (compare the radiolabel intensity of [35S]eNOS in lanes labeled 300 and 600 nM GST-dynamin, respectively, with lane labeled input (3 μl of reticulocyte lysate)). These observations are quantified in Fig. 4C, right panel, in which the densitometric intensity of the autoradiogram is depicted graphically. Conversely, incubation of GST-dynamin with increasing concentrations of [35S]-eNOS (1–12 μl of reticulocyte lysate) also results in a concentration-dependent binding between the proteins (data not shown). Note that the detected pools of eNOS and dynamin bound in vitro (Fig. 4, B and C) are significantly

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greater than that observed from cell lysates (Figs. 2C and 3). We next sought to quantify the binding affinity between eNOS and dynamin in vitro using radiolabeled tracer experiments and Scatchard analysis of binding data. 5.0 nM GST-dynamin was incubated with a logarithmic range of concentrations of purified recombinant eNOS protein (0–640 nM) premixed with proportional volumes of 35S-eNOS tracer. Bound radioactive counts were measured directly by scintillation counting in duplicate. In Fig. 4D, the Scatchard analysis demonstrates a K_d of 27.6 nM. To confirm that K_d estimation using this protocol is independent of the concentration of the immobilized protein, GST-dynamin, K_d calculations were performed at three different concentrations of GST-dynamin (5, 15, and 45 nM), each with saturating concentrations of eNOS (0–640 nM), with each experiment yielding similar K_d values. These studies indicate that dynamin-2 binds eNOS in a specific manner when the two proteins are maintained in solution. Additionally, these experiments indicate that in vitro, eNOS binds with dynamin in a direct manner with high affinity.

Dynamin Potentiates eNOS Catalysis—We next explored the biological significance of this novel protein-protein interaction between eNOS and dynamin-2. As ionophore stimulation of cells is associated with enhanced binding of dynamin and eNOS and, additionally, is known to increase NO release from cells is associated with enhanced binding of dynamin and eNOS and, additionally, is known to increase NO release from cells is associated with enhanced binding of dynamin and eNOS and, additionally, is known to increase NO release from cells is associated with enhanced binding of dynamin and eNOS and, additionally, is known to increase NO release from cells is associated with enhanced binding of dynamin and eNOS and, additionally, is known to increase NO release from cells is associated with enhanced binding of dynamin and eNOS and, additionally, is known to increase NO release from cells is associated with enhanced binding of dynamin and eNOS and, additionally, is known to increase NO release from cells is associated with enhanced binding of dynamin and eNOS and, additionally, is known to increase NO release from cells is associated with 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**DISCUSSION**

Several NOS-associated proteins have been identified that bind in a direct and specific manner with eNOS. Both calmodulin and Hsp 90 bind and directly activate eNOS, whereas the binding of caveolin and the bradykinin 2 receptor inhibit NOS activity (4, 6–9). The findings in this study indicate that dynamin-2 is a NOS-associated protein. We demonstrate a direct, specific, and high affinity interaction in vitro, which is regulated by ionic stringency and intracellular signals in vivo. Additionally, these studies provide evidence for a functional interaction as dynamin-2 augmenting eNOS catalysis in in vitro assays.
eNOS contains a unique amino-terminal consensus sequence, and acylation of this site targets the protein to membrane compartments including Golgi membranes and caveolae (1–3, 28). In prior studies, the distribution of dynamin protein within cells has been detected most prominently within Golgi membranes and associated clathrin-coated vesicles, as well as within the neck of endothelial cell caveolae as suggested by colocalization with the nascent Golgi protein, TGN 38 and with

**Fig. 4.** GST-dynamin and eNOS bind with high affinity *in vitro*. A demonstrates the purity and specificity of GST-dynamin-2. **Left**, a representative Coomassie-stained SDS-PAGE gel demonstrates the enrichment of a single 26-kDa protein from GST beads alone and the enrichment of a 130-kDa protein from GST-dynamin beads. **Right**, Western blot analysis demonstrates a specific signal for dynamin from GST-dynamin beads but not from GST beads alone. B, purified recombinant eNOS protein (60–300 nM) was incubated with GST-dynamin beads (150 nM) or, alternatively, with equal amounts of GST beads alone. Bound proteins were analyzed by Western blot analysis. **Left**, the representative blot demonstrates that GST-dynamin beads coprecipitate recombinant eNOS from solution, whereas GST beads alone do not coprecipitate eNOS from BAEC lysate. At a 1:1 molar ratio of GST-dynamin to eNOS (lane labeled 150 nM eNOS), a majority of the pool of available eNOS protein (lane labeled input, which represents the available recombinant eNOS used in the binding assay at a 1:1 molar ratio) is bound to dynamin. Further increase in the available concentration of eNOS (lane labeled 300 nM eNOS) increases binding only minimally (compare lane labeled 150 nM eNOS and lane labeled 300 nM eNOS). The blot is representative of experiments repeated five separate times with similar results. **Right**, densitometric intensity of the Western blot analysis is depicted graphically. Note that at a 1:1 molar ratio of eNOS and GST-dynamin (bar labeled 150 nM), the majority of available eNOS protein (bar labeled input) binds with dynamin. Results are the mean of duplicate densitometric determinations from the experiment shown on the left. C, GST-dynamin beads (60–600 nM) or GST beads alone were incubated with [35S]methionine-labeled eNOS transcribed *in vitro*. Binding of eNOS was analyzed by SDS-PAGE and autoradiography of dried gels. **Left**, the representative autoradiograph demonstrates that GST-dynamin binds [35S]-eNOS in solution in a concentration-dependent manner. The majority of the available [35S]-eNOS in solution (input, 3 µl of reticulocyte lysate) is bound to dynamin at concentrations of 300 and 600 nM GST-dynamin. Similar results were obtained in two independent experiments. **Right**, densitometric intensity of the autoradiograph is depicted graphically. Note that the majority of available eNOS (bar labeled input) is bound with GST-dynamin when present at a concentration of 300–600 nM. Results are the mean of duplicate densitometric determinations from the experiment shown on the left. D, a series of concentrations (0–320 nM) of purified recombinant eNOS protein, pre-mixed with proportionate amounts of [35S]-eNOS tracer (0–16 µl), were incubated with 5.0 nM GST-dynamin. Bound [35S]-eNOS and an [35S]-eNOS standard curve were analyzed directly by scintillation counting. Scatchard plot analysis is shown. Results represent an average of duplicate determinations from the same experiment, and the experiment shown is representative of two experiments that yielded very similar binding.
Dynamin and Endothelial Nitric-oxide Synthase

Dynamin potentiates eNOS catalysis in vitro and in cells. A, the catalytic activity of purified recombinant eNOS protein was examined after incubation with eluted GST protein, or after incubation with increasing molar concentrations of eluted GST-dynamin protein as described under “Experimental Procedures.” GST-dynamin significantly increases the enzymatic activity of purified recombinant eNOS as assessed by the l-NAME inhibited conversion of radiolabeled L-arginine to L-citrulline. (*, p < 0.05 versus GST + eNOS; **, p < 0.05 versus GST-dynamin + eNOS (0.5:1); ***, p < 0.05 one-way analysis of variance; GST-dynamin + eNOS (all concentrations) versus GST + eNOS; n = 3, each individual experiment performed in triplicate in the presence and absence of l-NAME). B, dynamin overexpression in cells potentiates NOS activity. eNOS-GFP ECV 304 cells were cotransfected with 6 μg of dynamin DNA, 6 μg of empty plasmid (pcDNA3) or, alternatively, 3 μg of each, and after 36 h, cell lysates were prepared for eNOS immunoprecipitation studies or, alternatively, NOS activity assay. Left panel, transfection of dynamin-2 DNA results in increased coprecipitation of dynamin with eNOS as shown in the representative Western blot (WB). Note that equal amounts of eNOS are immunoprecipitated from cell lysates in each group. Right panel, transfection of 6 μg of dynamin-2 DNA results in a significant increase in NOS activity (p < 0.05) as assessed by the l-NAME-inhibited conversion of L-arginine to L-citrulline in cell lysates. Transfection of 3 μg of dynamin DNA also increases NOS activity but not in a statistically significant manner (p > 0.05; not significant (NS)). Data shown are the mean and standard error from five separate experiments, each performed in duplicate.

caveolin, respectively (13, 14). In our studies eNOS and dynamin-2 colocalize predominantly in a perinuclear distribution in Clone 9 cells heterologously expressing both proteins as well as in BAEC, expressing both proteins endogenously. Additionally, the perinuclear distribution of both proteins colocalizes with the Golgi marker, Golgi 58-kDa protein, suggesting that within endothelial cells, endogenous eNOS and dynamin likely colocalize and interact within Golgi membranes. Thus functional eNOS protein interactions may not be exclusive to plasma membrane caveolae and may occur in other membrane compartments within which eNOS resides.

eNOS and dynamin-2 coinmunoprecipitates within cells, and additionally, dynamin in the form of a GST fusion protein coprecipitates both recombinant eNOS from solution as well as 35S-eNOS transcribed in vitro. Further specificity of binding is supported by a variety of experimental approaches including the demonstration of stoichiometric, high affinity binding kinetics in vitro, modulation of the interaction by ionic strength, and intracellular signals in vivo. The binding kinetics of eNOS and dynamin demonstrate interesting contrasts in vitro as compared with cell lysates. Our in vitro studies indicate that the recombinant proteins bind avidly in solution with an estimated K_0 of 27.6 nM. Of note, a previously published affinity analysis of dynamin with α-adaptin has demonstrated an estimated half-maximal binding of ~200 nM (20). In our studies, at a 1:1 molar ratio, purified recombinant eNOS protein in solution binds stoichiometrically with GST-dynamin. However, in vivo, tetrameric dynamin and dimeric eNOS may bind in an alternative kinetic manner. In our studies, significantly smaller pools of bound protein are detected from cell lysates by immunoprecipitation. Although the precise size of the bound pools of protein is confounded by detergents in the immunoprecipitation buffer which may disrupt protein interactions and thereby underestimate true binding, based on the signal intensity of dynamin bound to eNOS (IP) compared with the intensity of dynamin in the supernatant (Post IP) in Fig. 2, it would appear that ~5% of the pool of cellular dynamin is coprecipitated by eNOS in BAEC. Additionally, as demonstrated in the representative blot in Fig. 3, binding in response to ionomycin is increased suggesting that agonist stimulation is associated with enhanced binding of dynamin and eNOS. This degree of binding in cell lysates (5–10%) is similar to that observed with most NOS-associated proteins (7, 29–31), and therefore, it has been suggested that the interactions of eNOS with its associated proteins are more akin to transient signaling complexes rather than stoichiometric receptor-ligand type interactions (29). Additionally, with the varied cellular functions of dynamin, it would be unexpected for a large portion of the cellular dynamin pool to be bound to eNOS. Recent studies suggest that eNOS may reside in a complex in cells with other proteins including Hsp 90, caveolin, and calmodulin (5, 29). In this regard, our findings do not exclude the possibility that within cells binding of dynamin and eNOS is mediated in part through one or more of these proteins. In fact, as caveolin-1 binds directly with eNOS and additionally colocalizes with dynamin within cells (8, 13, 32), the inclusion of dynamin in such a protein complex is plausible. However in our studies, GST-dynamin binds recombinant eNOS and 35S-eNOS in vitro and potentiates eNOS catalysis in solution, indicating that the proteins can bind directly and in a functional manner in the absence of an adapter protein.

One of the primary cellular functions of dynamin relates to membrane fission events, in particular, vesicle budding and internalization of caveolae (12, 14). However, the dynamin family of proteins also participates in a variety of signaling cascades such as mitogen-activated protein kinase signaling, which are independent of their motor functions (15–17). Based on these divergent cellular actions of dynamins, one might postulate possible mechanisms by which dynamin-2 might impact on eNOS biology within the cell. One possibility is that the interaction of these proteins facilitates trafficking or translocation of eNOS and/or its associated signaling partners (33). eNOS resides within Golgi membranes and plasmalemmal caveolae, both compartments from which vesicles may bud in part through the scission actions of dynamin (1, 2, 12, 14). As eNOS undergoes dynamic movement between and within each of the two compartments (21, 34), the binding of the two proteins might facilitate the trafficking of eNOS within pools of vesicles liberated by dynamin. The present studies provide evidence for an additional mechanism that dynamin binds with...
eNOS and modulates NOS signaling and activity. This scenario is supported by the following: 1) the enhanced binding of dynamin with eNOS in cells treated with ionophore, a well-characterized agonist of NO production; 2) enhanced activity of purified eNOS protein in the presence of comparable molar concentrations of recombinant dynamin-2; and 3) enhanced binding of dynamin with eNOS and potentiation of eNOS catalytic activity in cells overexpressing dynamin-2. These findings suggest a novel role for dynamin-2 as a signaling protein for eNOS activation and are consistent with prior studies demonstrating the participation of the dynamins in a variety of signaling pathways (15–17, 35).

In summary, this study demonstrates that eNOS and dynamin bind in a direct, specific, and regulated manner and that binding results in an increase in eNOS catalysis. We anticipate that these events may have relevance for eNOS regulation and trafficking within the vascular endothelium.

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