

Receptor-regulated Translocation of Endothelial Nitric-oxide Synthase*

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The endothelial nitric-oxide synthase (eNOS) is activated by transient increases in intracellular Ca²⁺ elicited by stimulation of diverse receptors, including bradykinin B₂ receptors on endothelial cells. eNOS and B₂ receptors are targeted to specialized signal-transducing domains in the plasma membrane termed plasmalemmal caveolae. Targeting to caveolae facilitates eNOS activation following receptor stimulation, but in resting cells, eNOS is tonically inhibited by its interactions with caveolin, the scaffolding protein in caveolae. We used a quantitative approach exploiting immunofluorescence microscopy to investigate regulation of the subcellular distribution of eNOS in endothelial cells by bradykinin and Ca²⁺. In resting cells, most of the eNOS is localized at the cell membrane. However, within 5 min following addition of bradykinin, nearly all the eNOS translocates to structures in the cell cytosol; following more protracted incubations with bradykinin, most of the cytosolic enzyme subsequently translocates back to the cell membrane. The bradykinin-induced internalization of eNOS is completely abrogated by the intracellular Ca²⁺ chelator BAPTA; conversely, Ca²⁺-mobilizing drugs and agonists promote eNOS translocation. These results establish that eNOS targeting to the membrane is labile and is subject to receptor-regulated Ca²⁺-dependent reversible translocation, providing another point for regulation of NO-dependent signaling in the vascular endothelium.

translocation following their activation. Protein kinases, heterotrimeric G proteins, receptors, phospholipases; all these and many other signaling proteins may migrate from membrane to cytosol, from cytosol to membrane, or from one membrane compartment to another. For one protein, translocation may promote the desensitization of the signaling response; for another, subcellular translocation may be essential to fully activate the cell signaling pathway. These subcellular migrations may be modulated by reversible protein acylations, phosphorylations, protein-protein interactions, or ligand binding. The endothelial isoform of nitric-oxide synthase (eNOS)¹ can be modified by all of these processes; eNOS is regulated by both acylation and phosphorylation, and the protein reversibly interacts with disparate other proteins and ligands (for review, see Ref. 1). However, data on the role of the subcellular localization of eNOS in the enzyme's dynamic regulation remain controversial. Previous reports have assigned eNOS to the Golgi apparatus (2, 3), yet others have found the enzyme in cell membranes (4, 5) or exclusively in plasmalemmal caveolae (6, 7). Some investigators have failed to observe any agonist-promoted eNOS subcellular translocation (8), yet others have used biochemical approaches to suggest that the enzyme's targeting can be dynamically regulated (9–11). Using quantitative immunofluorescence imaging techniques, the current studies establish that eNOS is targeted to the cell membrane and that the enzyme's intracellular distribution is dynamically regulated by Ca²⁺-mobilizing agonists in vascular endothelial cells.

eNOS is a Ca²⁺-calmodulin-dependent enzyme and is transiently activated by increases in intracellular Ca²⁺ elicited by activation of diverse G protein-coupled receptors in endothelial cells and cardiac myocytes. Bradykinin represents one important endogenous activator of eNOS in the vascular wall. In the vascular endothelium, bradykinin binds to and stimulates Ca²⁺-mobilizing B₂ receptors, leading to vasorelaxation and to inhibition of platelet aggregation. eNOS undergoes acylation by the fatty acids myristate and palmitate; these modifications are required to target eNOS to the specialized signal-transducing domains in the plasma membrane termed caveolae. Other signaling proteins directly involved in eNOS regulation are also localized in caveolae, including the B₂ bradykinin receptor (12) and the m₂ muscarinic cholinergic receptor in cardiac myocytes. Targeting to caveolae thus appears to facilitate eNOS activation by these Ca²⁺-coupled signaling pathways.

In resting endothelial cells, the eNOS enzyme is tonically inhibited by its protein-protein interactions with caveolin, the resident scaffolding protein in plasmalemmal caveolae. In response to Ca²⁺-mobilizing agonists, calmodulin both promotes

A wide variety of signaling proteins undergo subcellular

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¹ The abbreviations used are: eNOS, endothelial nitric-oxide synthase; BAPTA/AM, 1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetra(acetoxymethyl) ester; BAEC, bovine aortic endothelial cells.

the dissociation of caveolin from eNOS and directly activates the enzyme. Biochemical approaches have also established that prolonged agonist treatment leads to translocation of a proportion of the eNOS protein from particulate to more soluble subcellular fractions, associated with phosphorylation (9) and depalmitoylation (13) of the enzyme. However, the cell biological correlates of agonist-induced translocation remain obscure, and the biochemical data are controversial. We have therefore explored the regulation of eNOS targeting using cellular imaging approaches and have discovered that the enzyme is reversibly targeted to endothelial cell membranes and that changes in intracellular Ca^{2+} represent a key determinant for eNOS redistribution.

MATERIALS AND METHODS

Reagents—Tissue culture reagents were from Life Technologies, Inc. eNOS monoclonal and caveolin polyclonal antibodies were from Transduction Laboratories. Secondary antibodies (rhodamine- or fluorescein-conjugated goat anti-mouse, rhodamine-conjugated goat anti-rabbit) were from Pierce. Fluo-3/AM was from Molecular Probes (Eugene, OR); BAPTA/AM was from Calbiochem; HOE140 was from Hoechst. Other reagents were from Sigma.

Endothelial Cell Culture and Drug Treatments—Bovine aortic endothelial cells (BAEC) were purchased from Cell Systems and maintained in culture in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin, and streptomycin (complete medium), as described previously (14). Thapsigargin, A23187, and BAPTA/AM were dissolved in Me_2SO , and cells were treated with thapsigargin (5 μM final concentration) and A23187 (10 μM) in Hanks' balanced salt solution with or without Ca^{2+} (1.5 mM) for 5 min at 37 °C. For studying effects of BAPTA, cells were preincubated with BAPTA/AM (20 μM final concentration) in complete medium at 37 °C for 30 min. The stock solution of bradykinin was prepared in phosphate buffered saline (PBS) and used at various final concentrations in complete medium at 37 °C.

Immunostaining—For cell imaging studies, BAEC were cultured onto gelatin-coated glass coverslips the day before analysis; cells were studied at passage 6. BAEC were fixed in 2% freshly prepared paraformaldehyde for 10 min at room temperature and permeabilized by adding (final concentrations) 0.1% Triton X-100 and 0.1% bovine serum albumin for 5 min at room temperature. The fixed cells were incubated for 1.5 h at room temperature (or overnight at 4 °C) in 10% goat serum and then with monoclonal anti-eNOS antibody at 1:250 dilution for 1 h at room temperature. The cells were then washed three times with PBS and then incubated with rhodamine-conjugated goat anti-mouse antibody at 1:200 dilution for 1 h at room temperature. A similar protocol was followed for the immunostaining of BAEC with Na^+/K^+ -ATPase and caveolin-1 antibodies.

Microscopy and Quantitative Analysis of eNOS Immunostaining—Cells were analyzed using a Zeiss epifluorescence microscope equipped with differential interference contrast optics. The cells were imaged at $\times 100$ – 1000 magnification using a charge-coupled device camera (model CH250, Photometrics Ltd.) cooled to -40 °C. We used conservative criteria to determine the presence of membrane-associated eNOS immunostaining. Since the membrane staining of eNOS was usually nonuniform in each individual cell, a cell was assigned as eNOS membrane-positive if even a small patch of membrane-associated eNOS was detected. Quantitative analyses of cells for the presence of membrane-associated eNOS were performed in a completely blinded fashion, after which the data were collated and normalized to the proportion of cells with membrane-associated eNOS under basal conditions. For each experiment at least 100 cells/coverslip were analyzed. Results are reported as an average of at least four independent blinded experiments performed in duplicate on different days; interexperimental variability was less than 5%.

Determination of Intracellular Calcium Concentration—Intracellular calcium determinations with the indicator dye Fluo-3 were performed as described previously (15). A stock solution of Fluo-3/AM, 1 mM in Me_2SO was diluted to the final concentration of 10 μM with Hanks' balanced salt solution. One ml of this solution was layered on a coverslip bearing BAEC, and cells were incubated for 60 min at room temperature in the dark. Cells were then washed three times with Hanks' balanced salt solution, sealed onto a custom microscopy chamber, and examined immediately. The chamber was designed to facilitate addition and removal of medium without disturbing the cells. A diamond pencil was used to cut two slivers of equal size from a No. 1 coverslip. The slivers were sealed on a glass slide with nail polish at the two longitu-

dinal edges, creating a channel in the center. Pressure was applied to ensure uniform sealing and leveling of the slivers. The coverslip bearing BAEC was inverted and sealed on the slivers with nail polish, forming a chamber that held approximately 100 μl of medium. The stock solution of bradykinin was diluted to 1 μM (final concentration) with complete medium and used immediately. A 1-ml tuberculin syringe containing bradykinin solution or medium alone was used to exchange at least 300 μl of medium rapidly, ensuring complete medium exchange for each experimental treatment. Epifluorescence microscopy was used to record fluorescence images of cells in real time after bradykinin treatment. Typically, six to eight cells were identified in a field of view ($\times 250$ magnification), and changes in the integrated fluorescence intensity of each cell were monitored over time. Image processing software (Metamorph, Universal Imaging, West Chester, PA) was used to draw cell boundaries and to integrate the fluorescence intensity over all pixels within the boundary of each cell. Calcium measurements were not affected by the exchange of drug-free medium alone.

RESULTS AND DISCUSSION

Immunostaining of BAEC using monoclonal eNOS antibody revealed a robust pattern of immunofluorescence (Fig. 1); when nonimmune mouse IgG1 was used as the primary antibody in identically processed samples, there was no staining (not shown). The cellular distribution of eNOS and caveolin immunoreactivity exhibited considerable heterogeneity. The majority of untreated endothelial cells (~ 60 – 80%) showed prominent eNOS immunostaining at the cell periphery; the remaining 20–40% of cells had no evident staining at the cell edge and showed eNOS distributed only in a patchy intracellular pattern with enhanced perinuclear staining (Fig. 1A). This patchy pattern of immunofluorescence has been observed for other caveolae-targeted proteins such as caveolin, cationic amino acid transporter protein, and c-Src, as well as prototypical "plasma membrane" receptors such as the cholecystokinin receptor and the high density lipoprotein receptor SR-B1 (16–20). The precise identity of the intracellular sites at which these proteins localize remains less well understood. As shown in Fig. 1C, caveolin and eNOS shared a similar but incompletely overlapping pattern of distribution in endothelial cells, with staining for the two proteins seen both at the plasma membrane and in similar intracellular locales.

The proportion of untreated cells without detectable eNOS at the membrane was variable from experiment to experiment (range 20–40%), but was quite consistent under identical experimental conditions within duplicate analyses. This heterogeneous pattern of membrane staining suggested that eNOS targeting may be influenced by cellular processes not definitively controlled in these experiments. Quantitative analyses of endothelial cells with any membrane-associated eNOS showed that $\sim 70\%$ of the cells' total immunoreactivity was present at the plasma membrane. Even within the positively staining endothelial cells, the membrane distribution of eNOS was usually nonuniform, and there were often large patches of eNOS-positive membrane located immediately adjacent to eNOS-negative regions (see Fig. 1). Importantly, when endothelial cells were treated with bradykinin (1 μM , 5 min), the pattern of eNOS immunostaining markedly changed (Fig. 1B); compared with untreated cells (Fig. 1A), BAEC treated with bradykinin showed a dramatic redistribution of eNOS from the cell membrane to a distinct ring pattern around the cell nucleus. Using similar immunofluorescence techniques, we found (data not shown) that the cellular distribution of other membrane-associated proteins (Na^+/K^+ -ATPase; caveolin) exhibited no cellular redistribution following bradykinin treatment.

The next series of experiments sought to validate the pharmacological characteristics of the bradykinin-induced eNOS redistribution. Given the heterogeneity of eNOS immunostaining within and between BAEC, we devised a conservative criterion for assessing whether eNOS translocation from the

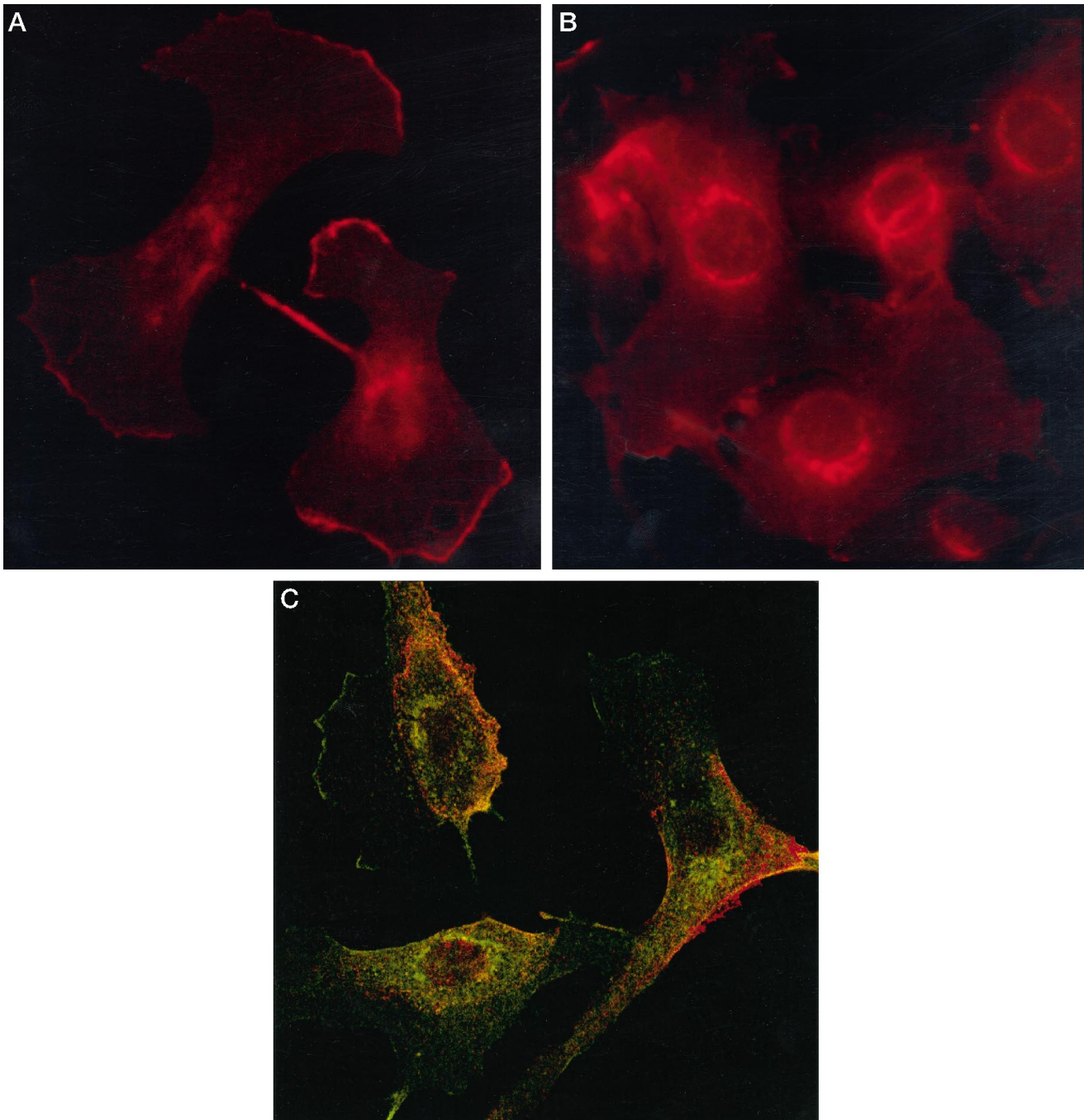


FIG. 1. Translocation of eNOS in endothelial cells in response to bradykinin. Shown are typical photomicrographs of bovine aortic endothelial cells processed for immunofluorescence as described in the text. *A* is a representative image of eNOS-immunolabeled BAEC studied under basal conditions. *B* is a representative image following treatment of endothelial cells with $1 \mu\text{M}$ bradykinin for 5 min and processing for eNOS immunostaining. Both images are at $\times 250$ magnification and are printed with identical brightness and contrast settings. *C* is a representative image showing the results of co-immunostaining endothelial cells, first with eNOS monoclonal antibody (secondary antibody conjugated to fluorescein, appearing as *green*) and then with caveolin polyclonal antibody (secondary antibody coupled to rhodamine, appearing in *red*); regions of eNOS-caveolin co-localization appear as *yellow*. The image in *C* was obtained using a Bio-Rad MRC-1024 confocal microscope.

membrane had taken place. Endothelial cells showing any identifiable membrane-associated eNOS immunostaining were scored as “membrane-positive”; only those cells completely devoid of membrane-associated eNOS were scored as negative. All samples were coded to permit the analyses to be performed and quantitated by a blinded observer unaware of the experimental treatment of the samples. Each experiment was performed in duplicate and repeated at least four times on different days using independently cultured cells. Because the proportion of cells scored as eNOS membrane-positive under basal conditions ranged from $\sim 60\%$ to more than 80% , for each

experiment results were normalized to the measured basal value on that day. For the analyses performed on any given day, duplicate values were reproducible within each experimental treatment; the percentage of cells positive for eNOS membrane targeting for any particular treatment varied by $<5\%$ between duplicate samples. Having validated the reproducibility and robustness of this experimental approach, we performed a dose-response analysis for bradykinin induced redistribution of eNOS (Fig. 2). After treating BAEC for 5 min with varying concentrations of bradykinin, the cells were fixed and immunostained, and the proportion of cells with mem-

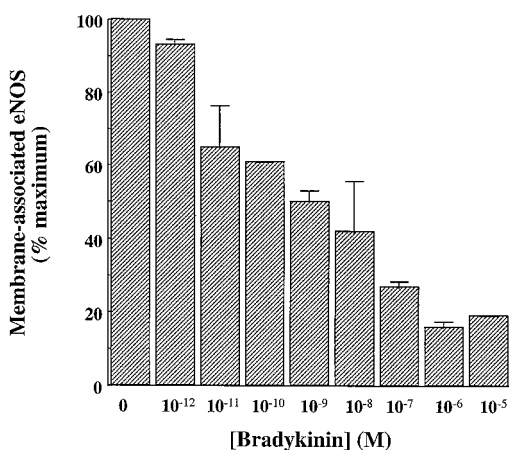


FIG. 2. eNOS translocation in endothelial cells: bradykinin dose-response. Bovine aortic endothelial cells were incubated with varying concentrations of bradykinin as shown, then fixed and processed for eNOS immunofluorescence as described under "Materials and Methods." The proportion of untreated cells with eNOS immunoreactivity at the cell membrane was determined, and this fraction was used to normalize the membrane-positive immunoreactivity observed at each concentration of drug. For each treatment, at least 100 cells were scored, and duplicate determinations were made for each dose; each data point represents mean \pm S.E. of at least four separate experiments.

brane-associated eNOS were quantitated. The EC_{50} for promotion of eNOS translocation by bradykinin was 1 nM; the maximum effect was observed at 1 μ M bradykinin. The B_2 -receptor antagonist HOE140 completely blocked bradykinin-induced eNOS redistribution; HOE140 alone did not affect eNOS distribution (data not shown).

The time courses of bradykinin-stimulated redistribution of eNOS and of bradykinin-stimulated changes in intracellular Ca^{2+} concentration are shown in Fig. 3, A and B, respectively. For immunofluorescence experiments, BAEC were treated with 1 μ M bradykinin for varying times, then fixed and processed as described above. For measurements of intracellular Ca^{2+} concentration, BAEC were first incubated with the indicator dye Fluo-3 for 60 min, then exposed to bradykinin (1 μ M) while determining the fluorescence emission of intracellular Fluo-3 at varying times. Treatment of endothelial cells with bradykinin resulted in a rapid loss of eNOS protein from the membrane; within 5 min, only $10 \pm 4\%$ of the BAEC originally showing eNOS membrane staining remained positive ($n = 25$). Upon prolonged incubation with bradykinin, the proportion of eNOS at the membrane gradually recovered; 60 min following addition of bradykinin, the proportion of cells with positive eNOS membrane staining reached $82 \pm 8\%$ of the value seen before the addition of drug ($n = 4$). The time course of the bradykinin-induced increase in intracellular calcium concentration ($[Ca^{2+}]_i$) followed a similar but not identical time course (Fig. 3B). There was a rapid initial rise of $[Ca^{2+}]_i$ nearly to maximal values within 1 min following the addition of bradykinin, followed by a slower increase of $[Ca^{2+}]_i$ over the next 20 min. The bradykinin-induced increase in $[Ca^{2+}]_i$ began to return to base line after 30 min following addition of bradykinin, associated with the recovery of membrane-associated eNOS.

The similar temporal patterns of bradykinin-induced changes in $[Ca^{2+}]_i$ and eNOS distribution led us to conduct a series of experiments to investigate further whether eNOS translocation might be mediated by changes in intracellular Ca^{2+} . In the presence of extracellular Ca^{2+} , the calcium ionophore A23187 promoted a rapid translocation of eNOS from the membrane (Fig. 4A). However, when A23187 was added to BAEC in the absence of extracellular Ca^{2+} , there was a signif-

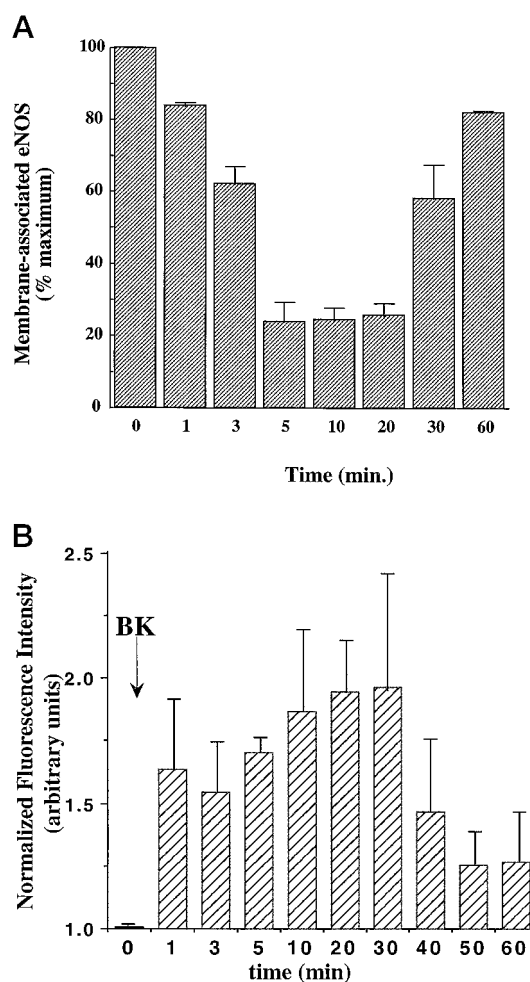


FIG. 3. Time course of bradykinin-induced eNOS translocation and alterations in intracellular Ca^{2+} . Bovine aortic endothelial cells were incubated for the indicated times with 1 μ M bradykinin, then fixed and processed for eNOS immunofluorescence (A) or analyzed using the calcium indicator dye Fluo-3 as described in the text (B). A, the proportion of untreated cells with eNOS immunoreactivity at the cell membrane was determined at $t = 0$ and at subsequent time points; this fraction was used to normalize the percentage of cells with membrane-positive immunoreactivity at each time point. For each time point, at least 100 cells were scored, and each treatment was analyzed in duplicate in at least four separate experiments; each data point represents mean \pm S.E. B, the integrated Fluo-3 fluorescence intensity for each cell (arbitrary units) was measured in the real time following bradykinin treatment and normalized by the fluorescence intensity for the cell before treatment. For each time point, 18 cells were monitored in three separate experiments; each data point represents mean \pm S.E.

icant and reproducible increase in the proportion of cells scored positive for eNOS membrane association. Presumably, this reflected the depletion of intracellular Ca^{2+} stores by the equilibration between intracellular Ca^{2+} and a large volume of extracellular Ca^{2+} -free medium. This interpretation was consistent with the effects of treating BAEC with thapsigargin, a specific inhibitor of the endoplasmic reticulum Ca^{2+} -ATPase, which is known to increase $[Ca^{2+}]_i$. As shown in Fig. 4A, addition of thapsigargin to endothelial cells promoted eNOS translocation from the membrane. In contrast, when BAEC were treated with A23187 plus thapsigargin in Ca^{2+} -free medium, there was an increase in the proportion of cells with membrane-targeted eNOS. These results indicate that the $[Ca^{2+}]_i$ plays a crucial role in the membrane targeting of eNOS.

Bradykinin B_2 receptors are coupled through G proteins to the activation of phospholipases C and A_2 (21), leading to the transient increases in $[Ca^{2+}]_i$ characteristic of the physiological

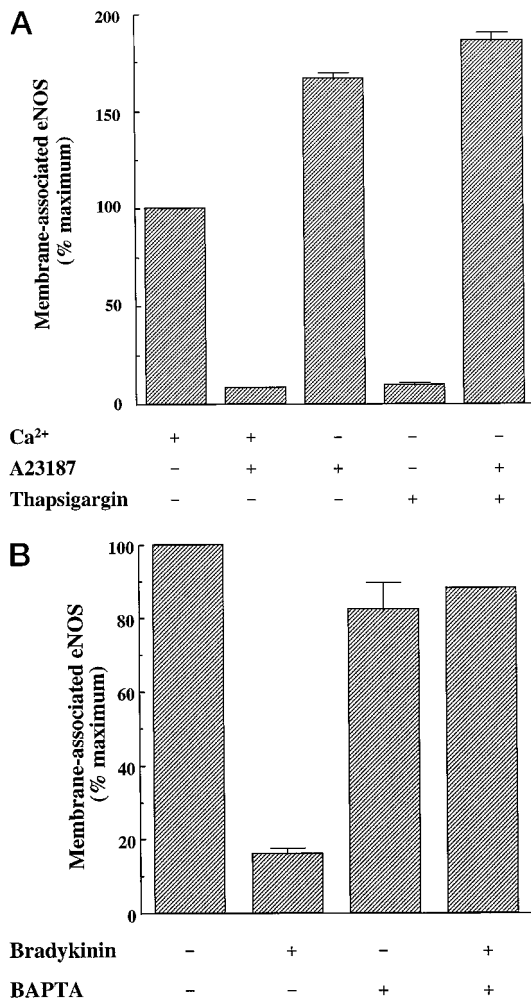


FIG. 4. Role of Ca²⁺ in dynamic targeting of eNOS. *A*, effects of calcium agonists. Cells were incubated with the calcium ionophore A23187 (10 μ M) and/or with thapsigargin (5 μ M) in the presence or absence of extracellular calcium (1.5 mM) for 5 min, then processed for immunostaining and quantitated for eNOS membrane targeting as described in the text. Each data point represents mean \pm S.E. of at least four separate experiments, each performed in duplicate. *B*, effects of intracellular Ca²⁺ chelation on bradykinin-induced eNOS redistribution. Cells were preincubated with BAPTA/AM (20 μ M) for 20 min at 37 $^{\circ}$ C, then stimulated for 5 min with 1 μ M bradykinin, and processed and analyzed for eNOS protein immunostaining. Each data point represents mean \pm S.E. of at least four separate experiments.

response to bradykinin (22). The influence of intracellular Ca²⁺ on eNOS translocation was explored further using the intracellular Ca²⁺ chelator BAPTA. As shown in Fig. 4*B*, preincubation of endothelial cells with BAPTA completely blocked bradykinin-induced eNOS redistribution. Our results thus suggest that increases in [Ca²⁺]_i may be both necessary and sufficient for eNOS translocation. It has been shown previously (23) that receptor-activated increases in intracellular calcium arise from regions of the endothelial cell membrane enriched in caveolin and quickly lead to increases in [Ca²⁺]_i throughout the cell. The nonuniform distribution of eNOS and caveolin along the endothelial cell membrane (Fig. 1) is reminiscent of the patchy membrane distribution described for caveolin (16, 23) and shown to correlate with regions of localized Ca²⁺-dependent signaling.

The dynamic equilibrium of eNOS membrane targeting in cultured endothelial cells is thus exquisitely sensitive to changes in intracellular calcium concentration. The heterogeneity of eNOS immunostaining, and its lability in the face of diverse agonists and under different conditions, may also help

to explain the discordance of our observations with several earlier reports. For example, Sessa *et al.* (2) and Liu *et al.* (3) failed to see any membrane-associated eNOS using immunofluorescence methods similar to those used in the current report; Venema *et al.* (24) visualized a small fraction of eNOS protein in endothelial membranes. The utilization of different antibodies and disparate culture conditions, the possibility of aberrant protein targeting in heterologous expression systems, untoward effects of growing cells in serum-free media; these may represent some of the confounding factors that could explain the negative findings of these earlier reports.

These studies also reveal a discrimination in the analysis of eNOS subcellular targeting as revealed by cellular imaging compared with biochemical approaches. When lysed endothelial cells are resolved into soluble and particulate preparations by ultracentrifugation, nearly all the eNOS enzyme is recovered in the particulate fraction. However, the particulate subcellular fraction, as simplistically defined by differential ultracentrifugation, has a heterogeneous composition. In the case of eNOS, the enzyme's targeting to the particulate fraction in resting endothelial cells reflects its association with intracellular membranes as well as cell surface structures. Following bradykinin treatment, essentially all the eNOS immunoreactivity is found around the circumference of the nucleus, possibly apposed to the nuclear membrane, raising interesting speculations about possible nuclear targets for NO following eNOS translocation.

The most plausible physiological consequence of eNOS redistribution following agonist activation is the enzyme's sequestration away from its proximal activators and concomitant attenuation of the extracellular release of NO. The time course of eNOS activation is much more rapid than the time course of enzyme translocation in the endothelial cell. Using a selective NO electrode, Malinski and Taha (25) found that bradykinin activates NO synthesis in endothelial cells within seconds of drug addition, with enzyme activity returning to base line in \sim 5 min. In contrast, bradykinin-induced translocation of eNOS is maximum only at 5 min and persists for several tens of minutes until the enzyme returns to the membrane (Fig. 3). These observations suggest that the activation and translocation processes reflect distinct components of the bradykinin response, with translocation plausibly representing a mechanism to down-regulate or uncouple enzyme activation from receptor occupancy. Taken together, our results establish that eNOS targeting to the cell membrane is labile and subject to receptor-regulated Ca²⁺-dependent reversible translocation, thereby providing another point for regulation of NO-dependent signaling in the vascular endothelium.

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