Mutation of N-Myristoylation Site Converts Endothelial Cell Nitric Oxide Synthase From a Membrane to a Cytosolic Protein

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Endothelial cell nitric oxide synthase (ECNOS) is a membrane-associated enzyme that generates endothelium-derived relaxing factor/nitric oxide (EDRF/NO) from L-arginine. We have suggested, from the cloning of the bovine ECNOS cDNA, that the presence of an N-myristoylation consensus sequence may impart its membrane localization since cytosolic forms of NOS do not contain such domains. To test the hypothesis that N-myristoylation is necessary for particulate ECNOS, we performed site-directed mutagenesis of the myristic acid acceptor site, Gly-2, and changed the glycine codon to alanine by a single nucleotide substitution. Expression of wild-type ECNOS in COS cells resulted in greater than 95% of the enzymatic activity in crude membrane fractions (as measured by the conversion of [3H]L-arginine to [3H]L-citrulline). In contrast, expression of the Gly-2 to Ala-2 mutant (G2A) demonstrated 8% ECNOS activity in membranes and 92% in the cytosol. The back mutation (from Ala-2 to Gly-2, A2G) restored ECNOS activity to the particulate fraction as seen with the wild type. Both wild-type membrane ECNOS and cytosolic G2A ECNOS activities were dependent on NADPH and calcium and were inhibited to the same extent by Nω-monomethyl L-arginine (L-NMMA) and Nω-nitro-L-arginine methyl ester (L-NAME). Moreover, kinetic analysis of these enzymes revealed similar Km for L-arginine (2–4 μM, n=3), demonstrating that the mutation did not affect ECNOS function. Thus, N-myristoylation is necessary for the membrane localization of ECNOS and may be of special significance for the basal or flow-induced production of NO by the endothelium. (Circulation Research 1995;72:921-924)

KEY WORDS • nitric oxide synthase • myristoylation • nitric oxide • site-directed mutagenesis

The nitric oxide synthase (NOS) family of proteins represents a unique class of mammalian enzymes that convert L-arginine to nitric oxide (NO) and L-citrulline. The novelty of these enzymes is their ability to catalyze the oxygenation of L-arginine (a cytochrome P-450–based reaction) and reduction of NADPH (a cytochrome P-450 reductase–like reaction) within the same protein, a dual attribute shared only by the bacterial flavoprotein Bacillus megaterium cytochrome P-450bsm,5

Three forms of NOS have been cloned to date: brain NOS (bNOS),4 macrophage NOS (MacNOS),6–8 and most recently, endothelial cell NOS (ECNOS).9–12 These enzymes share approximately 50% amino acid identity, are derived from separate genes, and are expressed in a cell-specific manner. bNOS and ECNOS are constitutively active, producing small quantities of NO when stimulated by calcium-mobilizing agonists, whereas MacNOS is induced by various cytokines, produces larger amounts of NO, and is not subject to regulation by calcium.13

In most endothelial cells, ECNOS is a membrane-associated enzyme, whereas bNOS and MacNOS are predominately cytosolic proteins.16,17 We and others10 suggested that the membrane association of ECNOS may be due to the presence of an N-myristoylation consensus sequence, a domain not present in soluble forms of NOS. Indeed, metabolic labeling of cultured endothelial cell with [3H]myristic acid demonstrated that semipurified ECNOS incorporates the labeled fatty acid.18 To test the hypothesis that N-myristoylation of ECNOS is necessary for its membrane localization, we performed site-directed mutagenesis of Gly-2, the myristic acid acceptor site found in N-myristoylated proteins and in ECNOS (but not in bNOS or MacNOS).

Materials and Methods

Site-Directed Mutagenesis of ECNOS

Oligonucleotide-mediated mutagenesis was performed with the Altered Sites mutagenesis kit (Promega) essentially as described by the manufacturer. In brief, the full-length ECNOS cDNA in Bluescript plasmid was digested with HindIII/XbaI and subcloned into the corresponding sites of the mutagenesis vector, pALT. Single-stranded DNA was made and annealed with mutagenic primers, and the complementary strand was synthesized. Primers were designed to introduce an
homogenized in a Dounce homogenizer and centrifuged at 10,000g for 45 minutes. Cytosolic fractions were passed over a Dowex AG 50WX-8 cation exchange resin to remove endogenous L-arginine. The 10,000g pellet and supernatants were then assayed for NOS activity. Membrane or cytosolic protein (50–100 μg) was incubated (total volume, 0.2 ml) in a 50 mM Tris-HCl/0.1 M EDTA/0.1 M EGTA buffer, pH 7.5, containing 1 mM NADPH, 3 μM tetrahydrobiopterin, 100 nM calmodulin, 2.5 mM CaCl₂, and 10 μM combined L-arginine and [³H]L-arginine (0.2 μCi; specific activity, 55 Ci/mmol) for 45 minutes at 37°C. An incubation time of 10 minutes was used in experiments examining the K₉ for L-arginine in ECNOS and G2A transfected cells. After the incubation period, the reaction was quenched by the addition of 1 ml of 20 mM HEPES stop buffer, pH 5.5 (containing 2 mM EDTA and 2 mM EGTA). The reaction mix was then passed over a 1-ml column containing Dowex AG 50WX-8 (Na⁺ form) resin (preequilibrated in stop buffer), was washed with 1 ml of water, and collected directly into a 20-ml liquid scintillation vial containing scintillation cocktail as described previously.⁹,¹⁰

Results

N-Myristoylation is a cotranslational modification that is important for protein localization and, thus, biological activity. In proteins demonstrated to be myristylated, the presence of a glycine residue next to the initiator methionine is required. After removal of methionine by an aminopeptidase, the terminal glycine then serves as the acceptor site for myristoyl-CoA.²⁰,²¹ For example, mutation of Gly-2 in tyrosine kinase p90⁶⁶ markedly interferes with its membrane localization and ability to transform cells.²²,²³ To test if the N-myristoylation consensus sequence site of ECNOS (MGXXXS) is of functional significance for the membrane association of this protein,⁹,¹⁰ we changed Gly-2 to Ala-2 by oligonucleotide-mediated mutagenesis.

Expression and Measurement of Nitric Oxide Synthase Activity

COS-1 cells were transfected with wild-type ECNOS, G2A, and A2G cDNAs (60 μg of plasmid DNA per 3×10⁶ cells in 150-mm dishes) by the DEAE-dextran method as described.¹⁹ Forty-eight to 72 hours after transfection, the cells were rinsed with cold phosphate-buffered saline and scraped into homogenization buffer (50 mM Tris-HCl/0.1 mM EDTA/0.1 mM EGTA, pH 7.5) containing the following protease inhibitors: 2 μM leupeptin/1 μM pepstatin A/2 μM bestatin and 1 mM phenylmethylsulfonyl fluoride. The cell suspension was

Let's start with the explanation of the results. The expression and measurement of nitric oxide synthase activity in COS-1 cells transfected with wild-type ECNOS, G2A, and A2G cDNAs were carried out. After transfection, the cells were harvested after 48 to 72 hours and rinsed with cold phosphate-buffered saline. They were then scraped into a homogenization buffer containing protease inhibitors and processed for measurement.

The results are as follows:

- **Expression and Measurement of Nitric Oxide Synthase Activity**
  - COS-1 cells were transfected with wild-type ECNOS, G2A, and A2G cDNAs (60 μg of plasmid DNA per 3×10⁶ cells in 150-mm dishes) by the DEAE-dextran method as described. After 48-72 hours, the cells were harvested, rinsed with cold phosphate-buffered saline, and homogenized. The homogenates were then analyzed for nitric oxide synthase activity.

**Results**

- **N-Myristoylation**
  - N-Myristoylation is a cotranslational modification that is important for protein localization and biological activity. The presence of a glycine residue next to the initiator methionine is required. After removal of methionine by an aminopeptidase, the terminal glycine serves as the acceptor site for myristoyl-CoA.

**Figure 1**

The nucleotide sequence of wild-type endothelial cell nitric oxide synthase (ECNOS) compared with the Gly-2 to Ala-2 mutant ECNOS (G2A) and the Ala-2 to Gly-2 back mutant ECNOS (A2G). The top figure is a representative autoradiogram from a DNA sequencing gel with a lane order (from left to right) of nucleotides a,c,g,t,a and a sense orientation (5′ bottom to 3′ top). Arrows on the left of ECNOS correspond to sequence from the initiator methionine (M) to Gly-2 (G). In the G2A and A2G mutations, arrows depict insertion of new Nco I site (ccagcg). On the bottom of the autoradiogram are the nucleotide sequences of wild-type, G2A, and A2G ECNOS showing nucleotides −8 to +4. Double-stranded DNA in pCDM8 was alkaline denatured and sequenced (Sequenase) with a T7 primer.

**Figure 2**

Expression of endothelial cell nitric oxide synthase (ECNOS) activity as indexed by the conversion of [³H]L-arginine to [³H]L-citrulline in membranes or cytosolic fractions prepared from transfected COS-1 cells. COS-1 cells were transfected with wild-type, Gly-2 to Ala-2 mutant (G2A), or Ala-2 to Gly-2 back mutant (A2G) ECNOS and cellular fractions prepared as described in “Materials and Methods.” Data are averages of duplicate incubations from a representative experiment.
DNA sequencing of the relevant regions of the G2A mutant revealed that the initiator methionine existed in the context of an Nco I site and the substitution of glycine codon (gcc) with alanine (gcc). In the back mutant (A2G), the substitution of alanine (gcc) to glycine (gcc) was accomplished (Figure 1).

Approximately 95% of ECNOS activity resides in the particulate fraction of membranes prepared from cultured or freshly isolated endothelial cells or ECNOS cDNA-transfected COS cells (Figure 2), with residual activity in the cytosolic fraction. Transfection of COS cells with the G2A mutant cDNA resulted in greater than 94% of ECNOS activity (n=3 separate transfections) in the cytosolic fraction (Figure 2). In contrast, transfection with the A2G cDNA restored the majority of NOS activity to the membrane fraction, as seen with expression of the wild-type ECNOS cDNA in COS cells. These results demonstrate that Gly-2 is necessary for targeting of ECNOS to its membrane compartment. In addition, 95% of bNOS activity (when subcloned into pCDM8 and transfected into COS cells) was localized as a cytosolic activity (n=2 transfections), demonstrating that the COS cell expression system provides the necessary translational events for NOS to be expressed as in native cells.

To assess whether the cytosolic G2A mutant protein is functionally the same as wild-type ECNOS, we characterized both enzyme activities further. Membrane (ECNOS) and cytosolic (G2A ECNOS) activities were attenuated by removal of calcium and NADPH from the incubation medium (Figure 3), both necessary factors for ECNOS activity. Furthermore, both enzymes were inhibited to the same extent by incubation with Nω-nitro-L-arginine-methyl ester (L-NAME, 100 μM) and Nω-monomethyl L-arginine (L-NMMA, 100 μM). The greater inhibition of wild-type and mutant (G2A) ECNOS activity by L-NAME is consistent with its

**Figure 3.** Characterization of endothelial cell nitric oxide synthase (ECNOS) activity in membranes or cytosol prepared from wild-type or Gly-2 to Ala-2 (G2A) ECNOS cDNA transfected COS-1 cells, respectively. Data are averages of duplicate incubations from a representative experiment.

**Figure 4.** Comparison of the kinetics of metabolism of [3H]l-arginine to [3H]l-citrulline of wild-type ECNOS membrane protein (○) and G2A ECNOS cytosolic protein (●). The top figure shows the substrate dependence of catalytic activity and the bottom a Lineweaver-Burk transformation of the data. The inset on the bottom figure shows the calculated apparent Km for l-arginine from the transformed data.
greater potency in vivo and in vitro. More importantly, the apparent $K_m$s for L-arginine were comparable (2.8 and 4.2 $\mu$M, $n=2$) in expressed wild-type (membrane) and G2A (cytosolic) ECNOS, respectively (Figure 4), showing that the mutation did not alter the conformation of ECNOS needed for L-arginine metabolism. These $K_m$s for L-arginine are similar to that reported for ECNOS purified from cultured bovine aortic endothelial cells (2.9 $\mu$M).15

### Discussion

The functional relevance of the membrane localization of ECNOS need not be clear. However, since N-myristoylation is a relatively rare modification that permits enzymes to be oriented near their endogenous substrates and to initiate or respond to compartmentalized cellular signals, it is possible that the membrane localization of ECNOS is significant. Of the five forms of NOS purified, ECNOS is the only major form that is membrane associated. A membrane-associated brain-derived NOS activity has been purified recently, but the nature of this enzyme is not known. Of the cloned cDNAs, only bovine9,10 and human ECNOS11,12 contain a consensus sequence for N-myristoylation.

The membrane localization of ECNOS may relate to the ability of endothelial cells to release NO in response to flow (and shear stress), as there is little evidence that other cells generate NO on mechanical stimulation (i.e., flow or shear). Although we have no direct experimental evidence as yet, it is possible that the membrane form of ECNOS (myristoylated) is situated near an L-arginine pool or is poised to respond to calcium released from sources close to the plasmalemma, whereas nonmyristoylated forms of ECNOS are not but can respond to various hormones that increase intracellular calcium. A corollary to this is that signals affecting the rate or extent of $N$-myristoylation may regulate ECNOS localization, and, hence, activity.

In summary, N-myristoylation appears necessary for the membrane localization of ECNOS. The generation of a soluble mutant form of ECNOS (G2A) that is otherwise indistinguishable from wild-type ECNOS will facilitate purification of the protein from transfected cells and allow us to study the regulation of membrane and soluble forms of ECNOS by hormones or shear stress.

### References

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