Docking of Endothelial Nitric Oxide Synthase (eNOS) to the Mitochondrial Outer Membrane

A PENTABASIC AMINO ACID SEQUENCE IN THE AUTOINHIBITORY DOMAIN OF eNOS TARGETS A PROTEINASE K-CLEAVABLE PEPTIDE ON THE CYTOPLASMIC FACE OF MITOCHONDRIA

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An association of NOS-like proteins with mitochondria has previously been demonstrated immunohistochemically (1–4). Further supporting evidence was provided by the partial purification of mitochondrial nitric oxide synthase (mtNOS) activity (5, 6). There is an ongoing debate as to the identity of mtNOS; immunologic cross-reaction of mtNOS with antibodies against endothelial NOS (eNOS) (1, 3, 7), neuronal NOS (4), and inducible NOS (6) have been reported. A recent investigation (8) has identified a neuronal NOS in cardiomyocyte mitochondria. It has been suggested that mtNOS plays important roles in oxidative stress and apoptosis (9, 10), regulation of mitochondrial respiration (11, 12), and modulation of intracellular Ca2+ homeostasis (13).

The potential distribution of mtNOS in subfractions of mitochondria has been explored but remains enigmatic. Some indirect evidence suggests that mtNOS is localized to the inner mitochondrial membrane. Indeed, immunohistochemical findings suggest that mtNOS co-localizes with succinate dehydrogenase, a mitochondrial marker for the inner membrane (2). Further support for an inner membrane localization of NOS in mitochondria came from NOS activity assays, which indicated that specific activity in submitochondrial particles and crude fractions was higher than that of mitochondrial homogenates or permeabilized mitochondria (14).

In contrast, a recent report (15) suggested that eNOS localizes to the outer membrane of mitochondria, as judged from immunoelectron microscopy of intact endothelial cells. In the present study, we sought to examine the molecular targeting of eNOS to mitochondria. Using three independent experimental approaches, we have verified that eNOS is present on mitochondria of HUVEC and specifically localized to the cytoplasmic face of the outer mitochondrial membrane. In vitro experiments with isolated mitochondria show that native and denatured eNOS interact with a proteinase K-hydrolyzable protein that participates in mtNOS recruitment to mitochondria. Deletion analysis revealed a stretch of five basic amino acids in the autoinhibitory domain (AID) of eNOS that is required for anchoring the enzyme to the mitochondrial outer membrane. Tethering of eNOS by its AID would predictably disinhibit enzymatic activity, allowing for NO synthesis by the mitochondrial membrane-bound eNOS. One of the consequences of the reduced mitochondrial association of AID-deleted eNOS is the increase in oxygen consumption.

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MATERIALS AND METHODS

Reagents and Antibodies—ATP, mannitol, phenylmethylsulfonyl fluoride, protease K, and nicotinamide adenine dinucleotide were obtained from Sigma. Precast gradient (20–30% Tris-glycine 500 × 500 mm) were obtained from Invitrogen. Mouse anti-eNOS monoclonal antibody was purchased from Transduction Laboratories (Los Angeles, CA). Mouse anti-human cytochrome oxidase subunit II monoclonal antibody, Alexa-Fluor goat anti-mouse IgG, and Alexa-Fluor goat anti-rabbit IgG were obtained from Molecular Probes (Eugene, OR). Rabbit anti-human eNOS and polyclonal antibody was obtained from Accurate Chemical and Scientific Corp. (Westbury, NY). Mouse anti-human VDAC antibody was obtained from BD Biosciences (Los Angeles, CA). Mouse anti-α-Na+/K+ A-subunit monoclonal antibody was purchased from Research Diagnostics, Inc. (Flanders, NJ). Mouse anti-idi- marker 58K protein monoclonal antibody was obtained from Abcam (UK). Rabbit anti-caveolin-1 polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse IgG horseradish peroxidase-conjugated whole antibody from sheep and anti-rabbit IgG horseradish peroxidase-conjugated whole antibody from donkey were obtained from Amersham Biosciences (UK). Peroxidase was from Amersham Biosciences (Piscataway, NJ), DMEM was from Biofluids (Rockville, MD), and EBM-2 was a product of Clonetics (San Diego, CA).

Cell Culture—Human umbilical vein endothelial cells (HUVEC) were grown in EBM-2 medium containing 2% fetal bovine serum, and used in experiments at the 6th passage.

Human embryonic kidney (HEK) cells and HEK cells stably transfected with a lacZ vector that encodes a stable expression of wild-type bovine eNOS (HEK-eNOS; a gift of Dr. William Sessa, Yale University School of Medicine) or a truncated enzyme lacking the autoinhibitory domain (HEK-ΔeNOS, 628–632 deletion) were cultured in DMEM containing 10% fetal bovine serum.

Construction, Propagation, and Purification of Adenoviral Vectors for Bovine eNOS (boNOS) Protein Expression—Construction of recombinant Ad-GFP-beNOS viruses was carried out according to the manufacturer’s protocol (Adeno-Quest, Quantum Biotechnologies Inc., Montreal, Canada). Briefly, CDNA-encoding bovine eNOS was subcloned into pQBIAd-GFP-IRES (lating the flanking BamHI sites eNOS to BglII cloning site on the vector). The vector carries an adenovirus origin of replication and packaging sequence, cytomegalovirus promoter, and an internal ribosome entry site element that provides expression of green fluorescent protein (GFP) and the inserted gene (wild-type eNOS or an eNOS deletion mutant lacking the oligonucleotide sequence that encodes amino acid residues 628–632, RRKKK) in a one-to-one ratio. The vector cloning site (BglII) is followed by a DNA sequence homologous to Ad5 9.4–15.5 map units. Recombinant adenovirus containing a homologous backbone is generated by cotransfection of the linearized plasmid vector with the QBI viral DNA into HEK-293A cells. Transfected 293A monolayers were overlaid with an agar-DMEM mixture and incubated at 37 °C with 5% CO2. Expression of GFP and the appearance of plaques were tracked by fluorescence microscopy, and plaques were isolated as soon as they were identifiable. Plaque lysates were subjected to three rounds of plaque purification, expanded on HEK 293A cell monolayers, and finally purified on SacIII step and continuous gradients, respectively. Viral titers ranged from 5 × 1012 viral particles per ml and were stored at –20 °C in a glycerol/BSA storage solution.

Adenoviral Infection of HEK Cells—HEK cells were plated in 10-cm plates to reach about 60–70% confluence at the time of transfection. The culture medium was removed, and 200 virus particles/cell were added to each plate in 2 ml serum-free DMEM. After incubation for 1 h at 37 °C with shaking, 8 ml of fresh complete DMEM were added. Cells were harvested for the isolation of mitochondria 6–24 h after infection.

Isolation of Mitochondria—All procedures were carried out at 4 °C unless otherwise specified. Mitochondria were isolated by differential centrifugation as described (16). Briefly, cells were washed twice with ice-cold PBS, scraped into MSH buffer (0.225 mol/liter mannitol, 0.75 mol/liter sucrose, 20 mmol/liter Hepes, pH 7.4) and homogenized with a Dounce homogenizer using 30 strokes. Cell homogenates were centrifuged at 600 × g for 10 min in a Beckman J-20 rotor, the pellet was discarded, and the supernatant was then centrifuged at 10,000 × g for 10 min. The pellet (crude mitochondria fraction) was washed with MSH buffer twice. Purified mitochondria were obtained by Percoll gradient centrifugation (16). Briefly, crude mitochondrial pellets were suspended in MEHB buffer (0.25 mol/liter mannitol, 1 mmol/liter EDTA, 5 mmol/liter HEPES, 0.1% (w/v) BSA, pH 7.4) and 100 μl was layered onto 3.0 ml of 30% (w/v) Percoll in 225 mmol/liter mannitol, 1 mmol/liter EDTA, 25 mmol/liter HEPES, 0.1% (w/v) BSA, pH 7.4, and spun at 95,000 × g for 30 min in a Beckman sw65 rotor. The brownish-yellow mitochondrial band was collected from the lower layer of the gradient, washed twice with MSH buffer to remove Percoll, and then suspended in a volume of MSH buffer to achieve a desired concentration.

Hypotonic Rupture of Mitochondria—Rupture of mitochondria was performed according to the protocol described by Geissler et al. (17). Mitochondria in MSH buffer were diluted with five volumes of 10 mmol/liter Hepes buffer, pH 7.4, to rupture the outer membrane by swelling. After 15 min of incubation on ice, an equal volume of 550H buffer (0.5 mol/liter sucrose, 10 mmol/liter Hepes, pH 7.4) was added to re-establish the original osmotic conditions. Mitochondria were then incubated by centrifugation and washed three times with MSH buffer.

Binding of Recombinant Bovine eNOS to Mitochondria—Purified mitochondria (10 μl, 2.5 mg/ml) and 90 μl of buffer (250 mmol/liter sucrose, 1% (w/v) fatty acid-free BSA, 80 mmol/liter KCl, 5 mmol/liter MgCl2, 2 mmol/liter ATP, 2 mmol/liter nicotinamide adenine dinucleotide, 1 mmol/liter diithiothreitol, 10 mmol/liter Mops-KOH, pH 7.2) were preincubated for 5 min at 25 °C before the addition of 1 μl of purified recombinant bovine eNOS (0.091 μmol/liter). After incubating at 25 °C for 1 h, 900 μl of MSH buffer were added to the samples. Mitochondria were subsequently re-isolated by centrifugation and washed twice with MSH buffer before protein extraction and analysis by SDS-PAGE.

Protease K Treatment—Mitochondria were treated with protease K at a final concentration of 50 μg/ml (from a 100× stock solution) for 25 min at 0 °C. Phenylmethanesulfonyl fluoride was then added to a final concentration of 2 mmol/liter, and samples were incubated for a further 10 min at 0 °C. Mitochondria were then pelleted by centrifugation.

SDS-PAGE and Western Blotting—SDS-PAGE was performed by using standard procedures on 4–20% Tris-glycine gels under reducing conditions. Proteins were electro-transferred to nitrocellulose and incubated with primary antibodies. The blotted bands were incubated with horseradish peroxidase-conjugated IgG and visualized with an enhanced chemiluminescence detection system (Pierce). Primary antibodies were used at the following dilutions: anti-eNOS antibody (1:500), anti-VDAC antibody (1:200), anti-fumarase antibody (1:1000), anti-cytochrome c oxidase (COX) antibody (1:1000), anti-cytochrome c antibody (1:200), and anti-caveolin antibody (1:1000). Secondary antibodies were used at a dilution of 1:1200.

Immunofluorescence—Mitochondria were incubated with 10% fetal bovine serum in PBS for 1.5 h and then treated with anti-eNOS monoclonal antibody at 1.200 dilution for 1 h at room temperature. Mitochondria were washed three times with PBS, incubated for 1 h with Alexa Fluor-goat anti-mouse antibody at 1:100 dilution at room temperature, and washed three times with PBS. Mitochondria were resuspended in PBS, and fluorescence was determined by using a fluorescence microplate reader (Molecular Devices) at excitation wavelength of 546/3 nm and emission wavelength of 580 nm. Specificity of fluorescence signal over mito-red was determined by double labeling with 30 μg/ml of goat anti-rabbit IgG. Mitochondria were observed in a Leica fluorescence microscope (JEOI, Ltd, Tokyo, Japan) operating at 80 kV.

Measurement of O2 Consumption—HEK cells were transfectioned with Adeno-eNOS, Adeno-ΔeNOS, and empty adenovirus vectors at their 80–90% confluency. Twenty-four hours later, cells were trypsinized, and the cell suspension with Krebs bicarbonate buffer (118 mm NaCl, 4.7 mm KCl, 1.5 mm CaCl2, 25 mm NaHCO3, 1.2 mm KH2PO4, 2.0 mm NaN3, 1.6 mm glucose, pH 7.4, containing 10 mm Hepes) and then suspended in the same buffer solution at a concentration of 1 million cells/ml. 3 ml cell suspension was then placed in a stirred chamber sealed with a Clark-type platinum O2 electrode (Yellow Springs Instruments, Yellow Springs, OH).
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RESULTS

Differential centrifugation of early passage HUVEC yielded several subcellular fractions that were probed for eNOS. Estimates from densitometric analysis of eNOS protein by Western blot analyses suggest that mitochondrial eNOS is robust, comprising ~15% of total whole-cell lysates (Fig. 1A). The band of eNOS disappeared after the mitochondria from HUVEC were treated with proteinase K (Fig. 1B, lanes 2 and 4), but was resistant to mitochondrial rupture by swelling (Fig. 1B, lane 3). Notably, proteinase K treatment has been used extensively to selectively remove proteins from the surface of mitochondrial outer membrane (18–20). To further analyze the sub mitochondrial localization of NOS in HUVEC, subfractions of mitochondria were identified by markers: VDAC for outer membrane (21), cytochrome c for intermembrane space (22), COX for inner membrane (16), and fumarase for matrix (16). The band on Western blots of the outer mitochondrial membrane marker VDAC was shifted to lower mass after treatment with proteinase K (lanes 2, 3), consistent with VDAC integration in the outer mitochondrial membrane. Mitochondria-associated eNOS, both native and denatured, was completely removed by treatment of incubates with proteinase K (lanes 2, 4). These data suggest that eNOS can bind selectively to the outer membrane of mitochondria. When the treatment of mitochondria with proteinase K was performed prior to the incubation with eNOS, eNOS binding was eliminated (lanes 5, 6), suggesting the existence of an eNOS binding protein(s) on the surface of mitochondrial outer membrane.

Experiments were performed to assess possible uptake of eNOS by mitochondria. Toward this end, purified mitochondria from Ad5-eNOS-infected cells were swollen in hypotonic medium, and subfractions were prepared that represent intermembrane space, inner membrane, and matrix. Results summarized in Fig. 4B demonstrate that the treatment of isolated mitochondria with proteinase K caused the disappearance of immunodetectable enzyme. Further, eNOS abundance in the swelling-ruptured mitochondria (in which the outer membrane was partially disrupted) was reduced compared with that present in an equal number of intact mitochondria.

Based on the above findings, one would predict that anti-eNOS antibodies should be able to efficiently recognize the bound eNOS, indicating that eNOS was not located in the matrix, inner mitochondrial membrane, or intermembrane space of mitochondria. These results suggest that eNOS is expressed exclusively on the surface of the outer mitochondrial membrane.

In the next series of experiments, we sought independent confirmation of eNOS localization to the outer mitochondrial membrane by immunoelectron microscopy (Fig. 2, A–F). In sample blanks (secondary antibody, but not primary), gold particles were not detected in association with purified mitochondria (not shown), whereas eNOS staining was consistently observed on the outer aspect of the outer membrane of mitochondria exposed to eNOS-specific primary antibody (Fig. 2, A–E). The pattern of eNOS distribution was patchy, as illustrated in the images presented. In accord with Western blot and immunofluorescence results, treatment with proteinase K consistently resulted in the disappearance of immunoelectron microscopy-detectable eNOS (Fig. 2F).

Next, we made use of a simple model system, wild-type HEK cells lacking eNOS, and infected the cells with adenoviral vector expressing eNOS protein. Appearance of eNOS protein in whole-cell lysates and mitochondrial fractions was monitored by Western blot analysis. Results of a representative experiment are illustrated in Fig. 3A. Initially, eNOS was undetectable in whole-cell lysates and isolated mitochondria. Six hours after Ad5-eNOS infection, trace amounts of eNOS became detectable, and at 10–12 h, eNOS expression was robust in both HEK cell lysates and in purified mitochondria. The purity of the isolated mitochondria was assessed based on the enrichment of a mitochondrial marker (COX) and the absence of protein markers for contaminating Golgi membranes (58K protein) or plasma membranes (Na+/K+ ATPase). Results suggest that the isolated mitochondria were 10-fold enriched in COX, but devoid of detectable Golgi membranes and plasma membranes (Fig. 3B). Collectively, these data suggest that newly synthesized eNOS associates with the mitochondrial fraction and that this cannot be attributed to contamination with Golgi or plasma membranes.

To examine the possibility of an import of eNOS from the cytoplasm, mitochondrial fractions obtained from wild-type HEK cells were incubated with the recombinant bovine eNOS (rbeNOS) in the next series of experiments. Results depicted in Fig. 4A demonstrate that rbeNOS can tightly associate with mitochondria in vitro (lane 1), and this association was not attenuated by prior denaturation of eNOS with 8 M urea (lane 3). Mitochondria-associated eNOS, both native and denatured, was completely removed by treatment of incubates with proteinase K (lanes 2, 4). These data suggest that eNOS can bind selectively to the outer membrane of mitochondria. When the treatment of mitochondria with proteinase K was performed prior to the incubation with eNOS, eNOS binding was eliminated (lanes 5, 6), suggesting the existence of an eNOS binding protein(s) on the surface of mitochondrial outer membrane.

Fig. 1. Expression of eNOS in the mitochondrial fraction of endothelial cells. A, analysis of subcellular eNOS abundance in HUVEC. Cell fractionation was performed as described under "Materials and Methods." An equal amount of protein was loaded per lane. Note that eNOS was detectable in the whole-cell lysate, plasma membrane, mitochondria, and Golgi apparatus. (Plasma membrane fraction is always contaminated with crude mitochondrial membranes.) Here and below, results are representative of at least three different experiments. Total, whole-cell lysate; PM, plasma membrane; Mito, mitochondria; ER, endoplasmic reticulum. B, treatment of mitochondria from HUVEC with proteinase K. Mitochondria were isolated from HUVEC and analyzed sequentially using the same detection chamber, and the variations of individual electrodes, samples from each set of cells were analyzed simultaneously using the same detection chamber, and the changes for each group (HEK/eNOS and HEK/ΔeNOS) were then averaged.
protein on non-permeabilized mitochondria. To examine this possibility, isolated mitochondria were incubated with anti-eNOS antibodies as detailed under “Materials and Methods,” followed by the detection of the antigen using fluorescence-tagged secondary antibodies. The intensity of eNOS immunofluorescence is quantified in Fig. 5. Mitochondria obtained from HEK-eNOS cells and HUVEC showed significant fluorescence intensity, far exceeding that of mitochondria obtained from wild-type HEK cells. Cells that were only exposed to secondary antibodies served as sample blanks. These data further support the view that eNOS antigen resides on the surface of mitochondrial membranes.

eNOS is unique among the NOS isoforms in its dual acylation by myristic and palmitic acids (25, 26). N-terminal myristoylation is required for the subcellular targeting of eNOS to cell membranes (where it associates with caveolin-1) and the Golgi complex (27). To address the possibility that acylation may similarly be necessary for the targeting of eNOS to mitochondria, a series of experiments were performed using an eNOS mutant defective in N-myristoylation. Mutation of gly-

![Image](https://example.com/image.png)

**Fig. 2.** Immunoelectron microscopy of eNOS localization in mitochondria isolated from HUVEC. A–E, representative images of mitochondria isolated from HUVEC stained with anti-eNOS antibodies (gold particles are indicated with arrows). These images represent different patterns of abundance of gold particles associated with the cytoplasmic face of the outer mitochondrial membrane, from robust to none. F, proteinase K treatment of isolated mitochondria virtually abolished outer membrane-associated staining. Magnification, ×19,000.
eNOS, treated with proteinase K as described under "Materials and Methods." Anti-eNOS antibody was used at a dilution of 1:5000, and anti-COX antibody was diluted 1:1000. Note the concomitant appearance of eNOS in whole-cell lysates and in mitochondria. B, the purity of mitochondria was tested with markers for different organelles by Western blotting. The marks used were COX for mitochondria, Na+/K+-ATPase for the plasma membrane, and 58K protein for the Golgi apparatus.

![Fig. 3. Mitochondrial eNOS in HEK cells.](image)

A, expression of eNOS in HEK cells. Cells were infected with adenovirus-containing eNOS gene and harvested after 6, 10, and 12 h. Mitochondria were isolated, subjected to SDS-PAGE (4–20% gel), and electro-transferred onto a nitrocellulose membrane. The expression of eNOS was analyzed by Western blotting as described under "Materials and Methods." Anti-eNOS antibody was used at a dilution of 1:5000, and anti-COX antibody was diluted 1:1000. Note the concomitant appearance of eNOS in whole-cell lysates and in mitochondria. B, the purity of mitochondria was tested with markers for different organelles by Western blotting. The marks used were COX for mitochondria, Na+/K+-ATPase for the plasma membrane, and 58K protein for the Golgi apparatus.

![Fig. 4. Localization of eNOS in the mitochondria.](image)

A, testing the possibility of the import of eNOS into mitochondria. Incubation of mitochondria from wild-type HEK cells with rbeNOS was performed as detailed under "Materials and Methods." Results of a representative Western blot: lane 1, rbeNOS + mitochondria; lane 2, the same as in lane 1 + treated with proteinase K; lane 3, urea-denatured rbeNOS + mitochondria; lane 4, the same as in lane 3 + treated with proteinase K; lane 5, proteinase K-treated mitochondria + rbeNOS; lane 6, proteinase K-treated mitochondria + urea-denatured rbeNOS; lane 7, non-treated mitochondria. B, treatment of mitochondria from HEK cells with proteinase K. Mitochondria were isolated from HEK cells transfected with eNOS, treated with proteinase K as described under "Materials and Methods" and analyzed by SDS-PAGE and Western blot analysis. Lane 1, intact mitochondria; lane 2, intact mitochondria treated with proteinase K; lane 3, swelling-shrinking-treated mitochondria; lane 4, swelling-shrinking of mitochondria with proteinase K treatment.

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cine-2 to alanine (G2A), the N-terminal myristoylation site of eNOS, did not attenuate the association of eNOS with mitochondria (Fig. 6A). These data indicate that myristoylation is not a prerequisite for localization of eNOS to mitochondria.

A stretch of five basic amino acid residues resides within the autoinhibitory domain of eNOS (residues 628–632 in bovine eNOS, RRKRK). Notably, this domain is a 45-amino acid peptide insertion in the FMN-containing domain that serves a physiological role in regulating eNOS activity (42). Limited trypsinolysis studies have indicated that the pentabasic sequence in the autoinhibitory domain is sequestered in quiescent enzyme, maintaining eNOS in an inactive conformation, and exposed after eNOS becomes activated subsequent to Ca²⁺/calmodulin binding (42). Interestingly, polybasic peptides such as HIV Tat and Antennapedia have been shown to interact with membrane glycosaminoglycans (43). To test the possibility that basic residues in the autoinhibitory domain of eNOS contribute to mitochondrial membrane anchoring, we contrasted binding of wild-type eNOS and ΔeNOS (deleted in RRKRK, eNOS628–632) to mitochondria in HEK cells, after infection with Ad5-eNOS or Ad5-ΔeNOS virus particles. As shown in Fig. 6B, deletion of the pentabasic amino acid sequence in the autoinhibitory domain resulted in a dramatic depletion of eNOS from mitochondria but did not interfere with plasmalemmal association.

To assess the potential functional significance of the finding of the wild-type eNOS associated with the cytoplasmic surface of the mitochondrion and the lack of such an association in cells expressing ΔeNOS, oxygen consumption was measured in HEK cells transfected with each of these constructs. As shown in Fig. 7, basal oxygen consumption was significantly increased in HEK-ΔeNOS cells compared with HEK-eNOS. Stimulation of eNOS with a calcium ionophore (A23187, 0.5 μg/ml) resulted in the decrease of oxygen consumption in both cell lines, although the preexisting difference remained. At higher concentrations of the ionophore (2.0 μg/ml), there was further inhibition of oxygen consumption in HEK-eNOS cells, but HEK-ΔeNOS chimeras failed to exhibit a more profound suppression of oxygen consumption. Both HEK/eNOS and HEK-ΔeNOS responded to the addition of a nitric oxide (NO) donor, soluble NSF attachment protein, with the same degree of inhibition of oxygen consumption, and both showed an increase in oxygen consump-

![Fig. 5. Immunofluorescence of isolated non-permeabilized mitochondrial eNOS from HEK cells, HEK-eNOS cells, and HUVEC.](image)

Mitochondrial eNOS in HEK cells. Planned mitochondria were incubated with anti-eNOS antibodies, which were developed using Alexa Fluor-conjugated secondary antibodies and quantified in a plate-reader, as detailed under "Materials and Methods." Blank (HEK control), primary antibody was omitted, and mitochondria were treated with secondary antibodies only. Excitation wavelength = 584 nm; emission wavelength = 612 nm.

![Fig. 6. Inability of eNOS lacking the pentabasic amino acid sequence from the autoinhibitory domain (628–632 amino acids) to bind to the mitochondria.](image)

A, expression of eNOS(G2A) in mitochondria obtained from HEK-eNOS(G2A) cells. Mitochondria were isolated from HEK cells stably transfected with eNOS(G2A). There was a robust immunodetectable eNOS band. B, the abundance of eNOS in the plasma membrane and in purified mitochondria of HEK/eNOS and HEK/ΔeNOS cells. An equal amount of protein was loaded per each lane for electrophoretic separation. eNOS lacking the pentabasic amino acid sequence was undetectable in mitochondrial fractions. Results are representative of three different experiments.
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FIG. 7. Oxygen consumption by HEK/eNOS versus HEK/ΔeNOS cells. The baseline oxygen consumption rate in HEK/eNOS was significantly lower than that of HEK/ΔeNOS (the actual rate of oxygen consumption is presented) but similar to that in HEK cells. Bars, oxygen consumption rate in HEK/eNOS and HEK/ΔeNOS after the consecutive addition of 0.5 µg/ml and 2 µg/ml A23187. A23187-induced decrease from the baseline in oxygen consumption in each cell population is shown in percentages: −25.8, −19.7, and −13.8% in HEK/eNOS, HEK/ΔeNOS, and HEK cells, respectively. Of note, A23187-induced decrease in oxygen consumption was significantly higher in HEK/eNOS and HEK/ΔeNOS cells compared with their HEK counterpart. About 3 million cells were used for each measurement. *, p < 0.01; †, p < 0.01 versus the corresponding experimental condition in HEK-eNOS cells; ‡, p < 0.05% change in oxygen consumption compared with HEK cells.

Discussion

In the present study, we provide several independent lines of evidence that eNOS in endothelial cells (or in chimeric cells infected with adenoviral eNOS) is located on the cytoplasmic face of the outer mitochondrial membrane: (i) we demonstrate that mitochondrial fraction of infection of wild-type HEK cells with eNOS-expressing adenoviral vector leads to the appearance of the enzyme in the mitochondrial fraction, concomitant with eNOS protein cellular expression; (ii) eNOS associated in vitro with mitochondria obtained from wild-type HEK cells; (iii) application of proteinase K not only completely removed immunodetectable eNOS from isolated mitochondria, but also prevented eNOS from binding to mitochondria in vitro. In addition, the antigen was found to be accessible to antibodies in non-permeabilized mitochondria. Finally, immunoelectron microscopy results confirmed the location of eNOS antigen on the outer mitochondrial membrane.

Mitochondrial respiration is under tonic inhibitory control of NO in accord with the finding that inhibition of NOS invariably increases mitochondrial oxygen consumption (28). Hence, insights into the topology of the enzyme in relation to the respiratory chain are crucial for understanding regulatory mechanisms of NO action. It has been reported that 5% of the oxygen consumed by the mitochondrial respiratory chain is converted into superoxide (29). The formed superoxide reacts with NO or could be converted to hydrogen peroxide (catalyzed by Mn-SOD, 10^8 mol liter^-1 s^-1; spontaneously, 10^5 mol liter^-1 s^-1) (30). NO is a lipophilic molecule and a highly diffusible free radical, the production of which is catalyzed by nitric oxide synthase from L-arginine (31). NO could react with superoxide and generate peroxynitrite at a rate of 10^7 mol liter^-1 s^-1 (32). The biotargets of peroxynitrite include heme-containing proteins, glutathione peroxidase (33), Mn-SOD (34), lipids (35), etc. Both Mn-SOD and glutathione peroxidase play an important role in preventing cells from reactive oxygen injury. Therefore, from the teleologic standpoint, the localization of eNOS to the cytoplasmic face of the outer mitochondrial membrane, where NO production would be spatially separated from superoxide generating systems, should be beneficial for the cell. Alternatively, the separation of NO and superoxide generating systems by the mitochondrial membranes could account for the graded and highly localized generation of nitric oxide and peroxynitrite, with the subsequent nitrosylation of COX and regulation of its function or irreversible inactivation of the enzyme, respectively.

Several lines of the evidence presented suggest that eNOS binds to the outer membrane in a manner distinct from its binding to the plasma membrane: it is independent of myristoylation, requires a proteinase K-cleavable partner, and shows discrete binding sites on the mitochondrial outer membrane. The fact that proteinase K treatment prevents eNOS association with mitochondria obtained from a non-endothelial cell line, HEK cells, argues that expression of the relevant binding protein is not restricted to endothelial cells. Caveolin, a scaffolding protein involved in organizing the distribution and activity of multiple signaling molecules in caveolae, has the capacity to interact directly with various intracellular proteins (25, 36, 37), including eNOS (with caveolin-1 and caveolin-3; Refs. 38–41). A recent study (37) reported that caveolin-1 is present in mitochondria. Although mitochondrial caveolin-1 was a plausible anchor for eNOS, the data obtained with HEK-ΔeNOS chimeras (prevention of mitochondrial binding of eNOS) did not support its involvement. On the other hand, the data demonstrated that the stretch of five basic amino acids within the autoinhibitory domain is required for anchoring eNOS to the outer mitochondrial membrane. One of the possible functional implications of the described topography of eNOS-mitochondrion association may be represented by the quality of controlling the oxygen consumption. As shown in Fig. 7, HEK-ΔeNOS chimeras exhibited a higher basal oxygen consumption and the less efficient suppression of oxygen consumption by elevated intracellular calcium, compared with the HEK cells transfected with wild-type eNOS. The fact that eNOS associates with the mitochondrion by means of its pentabasic sequence within the AID would argue that calmodulin is either already bound or would result in facilitated calmodulin binding (42). Indeed, we have previously demonstrated mitochondrial NO production by HEK cells transfected with wild-type eNOS (44). These observations argue in favor of a role played by the mitochondrion-associated eNOS in adjusting oxygen consumption under unstimulated conditions and a broader range of regulation in stimulated cells. Based on the findings presented, one would predict that the shuttling of a binding protein partner and eNOS in and out of the mitochondrial membrane could provide a powerful mechanism for locating NO bioactivity in mitochondria for control of mitochondrial respiration.

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Docking of Endothelial Nitric Oxide Synthase (eNOS) to the Mitochondrial Outer Membrane: A PENTABASIC AMINO ACID SEQUENCE IN THE AUTOINHIBITORY DOMAIN OF eNOS TARGETS A PROTEINASE K-CLEAVABLE PEPTIDE ON THE CYTOPLASMIC FACE OF MITOCHONDRIA

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