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HEME REQUIREMENT FOR PRODUCTION OF ACTIVE ENDOTHELIAL NITRIC OXIDE SYNTHASE IN BACULOVIRUS-INFECTED INSECT CELLS

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We have cloned cDNAs encoding endothelial nitric oxide synthase (ecNOS) from a human fetal liver cDNA library. Overproduction of ecNOS in a baculovirus/insect cell expression system in conventional medium yielded a large amount of ecNOS protein localized in particulate components, but ecNOS activity was low. This activity was increased by addition of hemin to 2.5-fold. While a precursor for heme biosynthesis increased the activity, inhibitors of heme biosynthesis reduced the ecNOS activity to 50% without affecting the level of enzyme. After extraction of cells with 1% Triton X-100, ecNOS protein was purified by column chromatography. The resultant ecNOS required supplementation with cofactors for activity, but it did not require hemin. Binding of a protoporphyrin IX heme was confirmed by a pyridine hemochrome assay.

The primary functions of NO produced by ecNOS in vascular endothelial cells may be to suppress platelet aggregation and to regulate blood pressure through relaxation of vascular smooth muscle cells as a result of activation of guanylate cyclase. In spite of their structural and catalytical similarity, the NOS isoforms appear to vary in their expression and regulation. The ecNOS is distinct from the other two isozymes in that its N-terminal amino

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Abbreviations: NO, nitric oxide; BH_4, tetrahydro-L-biopterin; bp, base pairs; DTT, dithiothreitol; NMA, N^G-methyl-L-arginine; NNA, N^G-nitro-L-arginine; NOS, nitric oxide synthase; bNOS, neuronal NOS; macNOS, macrophage NOS; ecNOS, endothelial NOS; PAGE, polyacrylamide gel electrophoresis; Sf21, *Spodoptera frugiperda* 21; PMSF, phenylmethanesulphonyl fluoride; BSA, bovine serum albumin; TBS, Tris-buffered saline, pH 7.4.

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acid is myristoylated and the enzyme is found in the particulate fraction (1). The activity of ecNOS and its intracellular localization seem to be regulated by phosphorylation of a particular serine residue in response to several physiological stimuli including bradykinin, sodium nitroprusside, and calcium ionophore (2). Because the content of ecNOS in endothelial cells is very low, purification and structure-function studies of the enzyme from this source are impractical. For this purpose, the baculovirus/insect cell system provides a powerful tool (3). This allowed spectrophotometric identification of a heme prosthetic group on the enzyme. The existence of a heme moiety in purified ecNOS was confirmed by formation of pyridine hemochromogen.

Materials and Methods

Cell culture
Culture of *Spodoptera frugiperda* (Sf21) cells and manipulation of baculovirus were carried out according to Piwnica-Worms (3). Sf21 cells were maintained in Grace's medium (GIBCO/BRL) containing 10% fetal calf serum, 3.3 mg/ml yeastolate, 3.3 mg/ml lactoalbumin hydrolysate, and 50 μg/ml gentamicin at 27°C.

Cloning of cDNAs and genomic DNA for human ecNOS
Two oligonucleotide primers, 5'-GGAATTCCACGCCTTCGATGCCAAG-3' and 5'-CAAGCTTTCGGTCATTGCTAATGAG-3', were used in a polymerase chain reaction with cDNAs obtained by reverse-transcription of rat cerebellar total RNA for bNOS. The PCR product (552 nucleotides) corresponding to nucleotides 2682-3234 of rat bNOS was purified by 1% agarose-gel electrophoresis and subcloned into Bluescript II KS$^+$ (Stratagene), and the sequence of the cloned PCR products was determined by the dideoxy chain termination method using the Sequenase version 2.0 kit (USB). This fragment was labeled with ^{32}P and used to screen a human fetal liver cDNA library (random and oligo (dT)-primed, Clonetech). The region encoding the 5'-end sequences of ecNOS missing in the human fetal liver cDNA was PCR-amplified from the human genomic DNA using the oligonucleotide primers 5'-ACGCACAGTAACATGGGCAAC-3' and 5'-TGTTCTGGCGCTGGTGGGAGTA-3'. The PCR product (170 nucleotides) corresponding to nucleotides 34-204 of human ecNOS was purified, subcloned, and sequenced as above. Wild-type cDNA for human ecNOS was constructed by ligating a restriction fragment (*Eco* RI/*Hha* I) of this PCR product to the cDNA from human fetal liver.

Construction of the recombinant baculovirus carrying human ecNOS cDNA
Human ecNOS cDNA was excised from the plasmid vector and was ligated into a baculovirus transfer vector, pVL1392 (Invitrogen). Foreign genes inserted into pVL1392 are expressed as nonfused proteins (4). The resulting plasmid was purified by cesium chloride ultracentrifugation, and 1 μg of plasmid DNA was cotransfected with baculovirus DNA (0.1 ng Baculogold, Pharmingen) into Sf21 cells using Lipofectin (Gibco BRL). The recombinant viruses were purified by a single round plaque assay and subjected to large-scale amplification. Viral infections were repeated a maximum of 4 times to avoid spontaneous mutation of the gene.

Overproduction and purification of ecNOS from post-infected Sf21 cells
10^6 Sf21 cells in a 3 cm dish were infected with baculovirus carrying human ecNOS cDNA at an infection multiplicity of 10 and were incubated for 3 days. Cofactors and their precursors in water were added directly to the medium after viral infection. The ecNOS produced by the Sf21 cells was purified from 10^8 cells using 2',5'-ADP-sepharose and

DEAE-cellulose. After extraction with 1% Triton X-100, all of chromatography was performed by the method described previously (5) except for containing 0.1% Triton X-100 in buffer.

Measurement of flavin and heme content
The heme content of the purified ecNOS was determined by measuring formation of pyridine hemochromogen (6). The heme concentration was calculated from the absorbance at 556 nm using an absorption coefficient determined with myoglobin as a standard. Spectral analyses were performed with a Beckman DU 7400 diode array spectrophotometer and 100 µl microcuvettes. All spectra were normalized to 0 absorbance at 700 nm. The flavin content was determined fluorometrically by reverse-phase HPLC (7).

Measurement of nitrite in the medium and NOS activity
Nitrite, the oxidized form of nitric oxide, that accumulated in the culture medium was determined spectrophotometrically using the Griess reaction (8). NOS activity was determined as the conversion of L-[guanidino-^{14}C] arginine to L-citrulline (9) with a minor modification (5).

Results

Cloning of ecNOS from a human fetal liver cDNA library - Messenger RNA from rat cerebellum was amplified by RT-PCR, and the resulting DNA fragments corresponding to bNOS were used to screen a human fetal liver cDNA library. Four independent, overlapping clones were isolated and sequenced. The isolated clones encoded human ecNOS and composed a 4070-nucleotide sequence including start and stop codons. Because the N-terminal 201 nucleotides of the 5'-most clone were completely different from those previously reported for ecNOS cDNA (10) and genomic sequences (11), we have PCR-amplified the 5'-end of the human gene, which is encoded in a single exon (11), and substituted this DNA for the aberrant portion of the 5'-end fragment to construct a full-length, wild-type ecNOS cDNA.

Overproduction of ecNOS in Sf21 cells - The full-length ecNOS cDNA was ligated into the pVL1392 vector. The recombinant baculoviruses extracted from three independent plaques all carried ecNOS cDNAs as judged by SDS-PAGE and NOS activity assays (data not shown). When the subcellular localization of ecNOS produced in infected Sf21 cells was examined by SDS-PAGE, the ecNOS protein was found in the particulate fraction after centrifugation of sonicated cells. This suggested that N-terminal myristoylation had occurred. To identify the ecNOS produced in Sf21 cells, we had performed SDS-PAGE (Fig. 1, Inset) and immunoblot (data not shown). Because the band of 140 kDa reacted with anti-human ecNOS antibody was not detected in uninfected cells, the 140 kDa protein was identified as a human ecNOS.

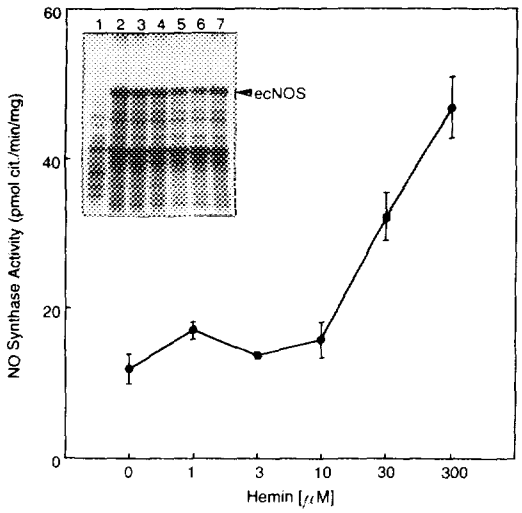


Figure 1. **Hemin dependency of the activity and expression levels of human ecNOS overproduced in Sf21 cells.**
Post-infected cells were incubated for 3 days in media supplemented with various concentrations of hemin. The activities of the homogenates were measured for three cultures at each point, and the mean values ± SD are presented. 10μg of proteins was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R-250. Lane 1, uninfected cells without hemin; lane 2, infected cells without hemin; lanes 3-7, infected cells with 1, 3, 10, 30, and 300 μM hemin, respectively.

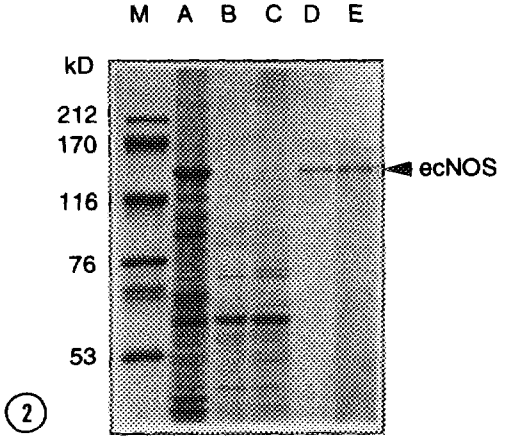
Heme requirement for ecNOS activity - Although ecNOS was produced in the post-infected insect cells at a very high level with the same molecular mass as mature protein, nitrite formation in the medium and NOS activity did not increase in proportion to the protein level when the cells were grown in conventional Grace's medium. While NOS activity and nitrite levels did not change in media supplemented with riboflavin, nicotinamide, BH_4, or L-arginine, supplementation with hemin brought about a marked increase in activity without increasing the level of ecNOS protein (Fig. 1). Hemin has also been shown to stimulate bNOS produced in a baculovirus/insect cell system (12). Because the solubility of hemin at neutral pH is low, its intracellular concentration would be much lower than shown in Fig. 1. Table I shows that 5 mM δ-levulinic acid, a precursor of heme, also stimulated nitrite formation and production of active ecNOS. Penicillamine and isonicotinic acid hydrazide, inhibitors of heme biosynthesis (13), suppressed both the accumulation of nitrite and the ecNOS activity without affecting ecNOS production (data not shown). This suggests that *de novo*-synthesized heme was used for formation of active ecNOS in the cells without hemin supplementation.

Table I. **Effects of hemin, a precursor, and inhibitors of heme biosynthesis on nitrite accumulation and NOS activity**

	NO_2^- (nmol/mg)	NOS activity (pmol cit./min/mg)
Uninfected cells	3.7 ± 0.8	1.1 ± 0.1
Infected cells		
Control	69.5 ± 5.6	44.0 ± 4.3
Hemin (50 μM)	142.5 ± 6.4	114.4 ± 14.8
δ-Aminolevulinic acid (5 mM)	123.1 ± 10.7	67.9 ± 1.4
Penicillamine (5 mM)	32.3 ± 4.7	24.5 ± 3.7
Isonicotinic acid hydrazide (5 mM)	36.9 ± 3.3	40.7 ± 2.2

After infection, cells were cultured with various compounds as shown in Fig. 1. Nitrite levels and NOS activities were measured in triplicate experiments, and mean values ± SD are presented.

Purification and characterization of ecNOS - To further characterize the ecNOS produced in Sf21 cells, the protein was purified by chromatography on 2',5'-ADP-sepharose and DEAE-cellulose. Fig. 2 shows the results of SDS-PAGE after each step of purification. Approximately 250 μg of ecNOS protein was recovered from 1 x 10^8 cells with specific



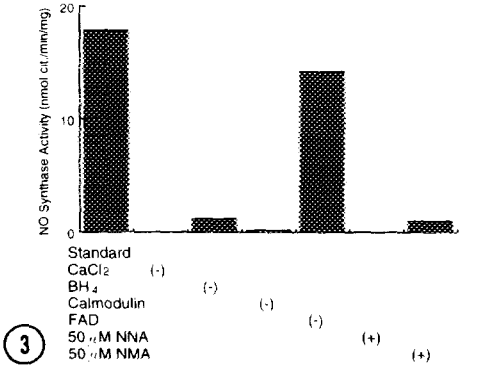


Figure 2. **SDS-PAGE analysis of proteins at each stage of purification.** All proteins were reduced and analyzed by 7.5% SDS-PAGE followed by staining with Coomassie Brilliant Blue R-250. Lane A, homogenate (20 μg); lane B, the supernatant after ultracentrifugation at 105,000 x g for 1h (10 μg); lane C, 1% Triton X-100 extract (10 μg); lane D, the eluate from 2',5'-ADP sepharose (2 μg); lane E, the protein purified by DEAE-cellulose (1 μg); lane M, molecular weight markers.

Figure 3. **Cofactor requirement for ecNOS activity and the effect of inhibitors on purified enzyme.** One cofactor at a time was replaced with H_2O (-) in the standard reaction mixture. In inhibition assays, the concentration of inhibitor was 50 μM (+). The means of duplicate measurements are given.

activity of 17.9 nmol/min/mg. We also examined cofactor requirements and the effect of inhibitors on ecNOS activity using purified enzyme (Fig. 3). Calcium, BH_4, and calmodulin were required for full enzymatic activity *in vitro*, whereas substantial activity remained in the absence of added FAD. The bound heme was sufficient for expressing the ecNOS activity.

Determination of heme and flavin content - The absorbance spectrum of the purified ecNOS is shown in Fig. 4A. The spectrum of resting NOS exhibited a broad range of absorbance near 395 nm with a shoulder between 440 and 500 nm. This shoulder was derived from enzyme-bound FAD and FMN and was also observed in bNOS and macNOS (14, 15). The strong absorption at wavelengths under 360 nm was due to the high concentration of Triton X-100 after concentration of the sample using Centricon. To identify the bound heme, the pyridine hemochromogen method (6) was used. As shown in Fig. 4B, the pyridine hemochromogen spectrum of ecNOS in the presence of 0.1 N KOH and 30%

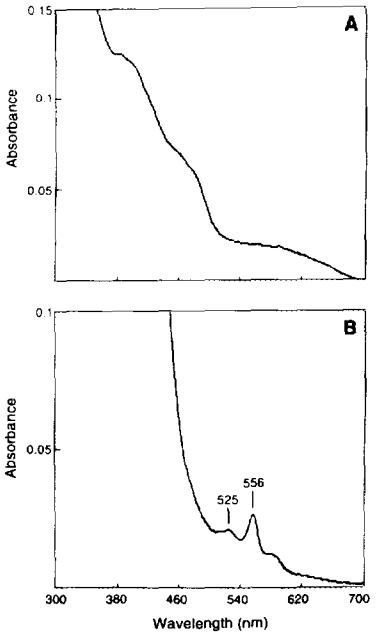


Figure 4. **Absorbance spectra of purified ecNOS.**
(A) Absolute spectrum. The spectrum represents resting ecNOS purified in the absence of L-arginine. (B) Spectrum of reduced pyridine hemochromogen. The spectrum of pyridine hemochromogen was obtained with a 100-µl aliquot of ecNOS in the presence of 0.1 M KOH and 30% (v/v) pyridine after addition of a few grains of sodium dithionite. Maximal absorption was obtained after 2 min.

pyridine after addition of sodium dithionite showed characteristic absorbance bands at 525 and 556 nm as reported for the pyridine hemochromogen spectra of ferroprotoporphyrin IX-containing proteins (16, 17). The amount of heme bound to ecNOS was calculated from its pyridine hemochromogen spectrum using myoglobin as a standard. Using a standard absorption coefficient of 0.019 mM^{-1} at 556 nm and a molecular mass of about 140,000 daltons for the ecNOS monomer, the molar ratios of heme, FAD, and FMN were calculated to be 0.79, 0.7, and 0.82 mol per monomer ecNOS, respectively.

Discussion

We have demonstrated a heme requirement for the production of active ecNOS using a baculovirus/insect cell system and have shown stoichiometric binding of heme and flavins to the purified recombinant ecNOS. Although spectrophotometric studies have identified heme in purified bNOS and macNOS (14, 15, 18, 19), no direct evidence was available for ecNOS. In addition, while FMN, FAD, NADPH, BH_4, and Ca^{2+}/calmodulin are known to be required for catalytic activity (20), whether heme plays a role in catalysis has been unclear. The involvement of the heme moiety in NO synthesis was studied by measuring nitrite accumulation in the culture medium and NOS activity when cells cultured in the presence of hemin, a precursor, and inhibitors of heme biosynthesis. As shown in Table I, addition of hemin or a heme precursor increased nitrite and citrulline formation. Addition of penicillamine and isonicotinic acid hydrazide, inhibitors of heme biosynthesis, resulted in 50% inhibition of nitrite and citrulline formation. These data indicate that binding of heme to NOS is essential for the enzymatic conversion of L-arginine into L-citrulline and NO. NO is formed by NOS by a rather complex series of reactions that constitute an oxidation involving five electrons (20). Thus, ecNOS might contain an oxygenase domain as found in cytochrome P-450 and a reductase domain containing binding sites for flavin nucleotides.

Recently Chen *et al.* (21) reported that the heme binding sites of ecNOS are Cys-99 and Cys-184 by site-directed mutagenesis. They found that the mutants with amino acid substitutions in these residues had no activity and exhibited no CO different spectra by using soluble fractions prepared from baculovirus-infected Sf9 cells. Eventhough the expressed enzyme consituted 50% of the total soluble proteins, the NOS activity was much less than the expected value from the protein level, indicating that most of the enzyme

existed as an inactive form. Here we provided convincing evidence that heme is essential component for ecNOS activity. Although they could not find stimulation of NOS activity by addition of hemin to the culture medium, hemin actually stimulated the activity to 2.5-fold in our system (Fig. 1). Data using a precursor or inhibitors of heme biosynthesis (Table I) also indicated that *de novo* synthesis of heme was essential for production of active ecNOS in the cells. Furthermore, we purified ecNOS from infected insect cells (Fig. 2) and directly demonstrated the equimolar binding of protoporphyrin IX (Fig. 4), FAD, and FMN per monomer of the purified enzyme. Inhibition of NOS activity by NO itself (15, 22), therefore, could be due to the binding of NO by the ferric-iron of the heme localized at the catalytic center of the oxygenase domain. The availability of a large amount of active ecNOS will facilitate structure-function studies of the enzyme and the development of drugs useful for clinical purposes.

Acknowledgments

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