BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **226,** 293–300 (1996)
ARTICLE NO. 1348

Reductase Domain Cysteines 1048 and 1114 Are Critical for Catalytic Activity of Human Endothelial Cell Nitric Oxide Synthase as Probed by Site-Directed Mutagenesis

J. L. Zhang, J. M. Patel,[1] Y. D. Li, and E. R. Block

Department of Medicine, University of Florida and VA Medical Center, Gainesville, Florida 32608-1197

Received July 24, 1996

We examined whether highly conserved cysteine residues in the reductase domain of the constitutive isoform of nitric oxide synthase in human endothelial cells (ecNOS) are crucial for catalytic activity of the enzyme. Substitution of alanine for cysteines 976 (Cys-976), 991 (Cys-991), 1048 (Cys-1048), or 1114 (Cys-1114), located in the reductase domain of human ecNOS, was achieved by oligonucleotide-directed mutagenesis and expression in COS-7 cells. The specific activity of ecNOS was >7-fold increased in wild-type and in mutants Cys-976 and Cys-991, but not in mutants Cys-1048 and Cys-1114. However, Western blot analysis indicated that expression of ecNOS protein was comparable in wild-type and in all mutants. NADPH concentration-dependent L-citrulline formation and NADPH oxidation during L-arginine metabolism were reduced in mutants Cys-1048 and Cys-1114 compared to wild-type. Similarly, NADPH cytochrome c reductase activity was increased in a time-dependent fashion in wild-type but not in mutants Cys-1048 and Cys-1114. These results indicate that Cys-1048 and Cys-1114 residues in the NADPH binding site of the reductase domain are critical for human ecNOS activity. The lack of utilization of NADPH in L-arginine metabolism and in cytochrome c reduction suggests that these active site cysteine residues may be responsible for binding of NADPH and/or for electron transfer in human ecNOS.

Nitric oxide synthase (NOS), a novel family of enzymes containing heme oxygenase and cytochrome P-450 reductase domains, catalyzes the oxidative metabolism of L-arginine to generate nitric oxide (NO) by vascular endothelial cells (1,2). A major portion (>90%) of the constitutive isoform of endothelial cell NOS (ecNOS) is in the membrane fraction, and its catalytic activity is calcium-, calmodulin-, tetrahydrobiopterin-, and nicotinamide adenine dinucleotide phosphate (NADPH)-dependent (3,4). The molecular cloning and characterization of isoforms of NOS proteins from various organs and diverse species reveal the presence of similar structural and functional domains with 60-90% amino acid sequence identity and the presence of >25 cysteinyl residues (4). The oxygenase domain of NOS proteins contains heme, tetrahydrobiopterin, and L-arginine binding sites, whereas the reductase domain of NOS contains NADPH and flavin binding sites by sequence homology to cytochrome P-450 reductase. The oxygenase and reductase domains are linked by a calcium-calmodulin binding site and are believed to play a critical role in facilitating electron transfer from NADPH in the reductase domain to the oxygenase domain, resulting in oxidative metabolism of L-arginine to NO (5,6).

Recent reports have demonstrated that structural modulation of active site cysteines 184 and 99 in the oxygenase domain results in the loss of catalytic activity of human ecNOS (7,8). We have recently reported that chemicals that specifically react with protein thiols, but not heme, reduce the catalytic activity of porcine ecNOS (9), indicating that the cysteinyl residues

[1] To whom correspondence should be addressed: Research Service (151), VA Medical Center, Gainesville, FL 32608-1197. Fax: (352) 374-6170.

0006-291X/96 $18.00

TABLE 1
Alignment of Amino Acid Sequences of the Reductase Domain of NOS Isozymes

	FAD	NADPH
Human ecNOS (20)	^{969}GPLHYGVCSTWLSQLKPGDPVPCFIRGAPS^{998}	.....^{1043}TLVFGCRCPQ^{1052}.....^{1109}GHMFVCGDVT^{1118}
Bovine ecNOS (21)	^{971}GPLHYGVCSTWLSQLKTGDPVPCFIRGAPS^{1000}	.....^{1045}TLVFGCRCPQ^{1054}.....^{1111}GHMFVCGDVT^{1120}
Human iNOS (22)	^{937}GPLHHGVCSTWLNSLKPQDPVPCFVRNASA^{966}	.....^{1011}TLVFGCRRPD^{1020}.....^{1077}GHLYVCGDVR^{1086}
Mouse iNOS (23)	^{931}GPLHHGVCSTWIRNLKPQDPVPCFVRSVSG^{960}	.....^{1005}SLVFGCRHPE^{1014}.....^{1071}GHLYICGDVR^{1080}
Rat iNOS (24)	^{934}GPLHHGVCSTWINNLKPEDPVPCFVRSVSA^{963}	.....^{1148}SLVFGCRHPE^{1157}.....^{1074}GHLYICGDVR^{1083}
Human nNOS (25)	^{1208}GPIHHGVCSSWLNRIQADELVPCFVRGAPS^{1237}	.....^{1282}VLVFGCRGSK^{1291}.....^{1348}GHIYICGDVR^{1357}
Rat nNOS (16)	^{1204}GPVHHGVCSSWLNRIQADDVVPCFVRGAPS^{1233}	.....^{1278}VLVFGCRGSK^{1287}.....^{1344}GHIYICGDVR^{1353}

The corresponding amino acid sequences of the FAD-NADPH binding sites of the reductase domain of NOS isozymes: human and bovine ecNOS, human, mouse, and rat inducible NOS (iNOS), and human and rat neuronal NOS (nNOS). Numbers in parentheses are reference citations. Underlined sequences represent conserved regions.

located outside of the heme binding site of this protein are also critical for its catalytic activity. Amino acid sequence comparison of the reductase domain of all known NOS isoforms reveals the presence of highly conserved cysteinyl residues containing consensus sequence motifs xGVCSx, xVPCFx, xLVFGCRx, or xCGDVx as shown in Table 1. Because the functional role of the reductase domain is to facilitate electron transfer and activate oxygen in the oxygenase domain, the present study was designed to test the hypothesis that mutation of cysteinyl residues in the flavin adenine dinucleotide (FAD)-NADPH binding sites of the reductase domain causes alterations in the catalytic activity of human ecNOS.

METHODS

Chemicals. Calmodulin, leupeptin, cytochrome c, EDTA, EGTA, phenylmethylsulfonyl fluoride (PMSF), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), and N-[2-hydroxyethyl]piperazine-N′-[2-ethansulfonic acid] (HEPES) were purchased from Sigma Chemicals (St.Louis, MO). Tetrahydrobiopterin was purchased from Dr. B. Schirck's laboratory (Jona, Switzerland). Dowex AG1-X8 (hydroxide form, 100-200 mesh), AG50W X8 (sodium form, 200-400 mesh), and unlabeled L-arginine were obtained from Bio-Rad (Richmond, CA).

Cell culture. COS-7-SV 40 transformed, African green monkey, kidney cells obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO) and antibiotics were used for all experiments.

Cloning of human ecNOS and oligonucleotide-directed mutagenesis. Human ecNOS cDNA (kindly provided by Dr. Philip A. Marsden, University of Toronto, Canada) was cloned into a mammalian expression vector-pcDNA3 (Invitrogen, San Diego, CA) in sense orientation to form pHEN20 and in antisense orientation to form pHEN8. pHEN20 and pHEN8 were used for expression in COS-7 cells as wild-type ecNOS and negative control (i.e., antisense), respectively. Single-stranded DNA in sense orientation to pHEN20 was purified, and antisense oligonucleotide primers (AG CCA CGT GGA **GGC** CAC TCC ATA GT), (CC CCG GAT GAA **GGC** CGG CAC AGG GT), (TG GGA GCA TCG **GGC** GCC GAA CAC CA), or (GT AAC ATC GCC **GGC** CAC AAA CAT GT) were designed for mutation of Cys-976, Cys-991, Cys-1048, or Cys-1114 to alanines (bold letters) and for inclusion of Hae III, Nae I, Nar I and Nae I restriction enzyme sites (underlined letters), respectively. These oligonucleotide primers were synthesized by the DNA Core Facility of the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida. Single-stranded DNA (90.05 pmol) and 1.25 pmol of phosphorylated oligonucleotide primers Cys-976, Cys-991, Cys-1048, or Cys-1114 were annealed, and then the mutant strand was synthesized. The mutant DNA was transformed into ES1301-competent cells (mutS), a mismatch repair minus strain of E. coli (Promega, Madison, WI). Plasmid DNA was purified from the ES1301 cells and then transformed into JM109 cells (Promega). Colonies were prescreened by plasmid miniprep and Hae III, Nae I, or Nar I digestion. DNAs mutated at Cys-976, Cys-991, Cys-1048, or Cys-1114 (cysteine to alanine) were selected and further confirmed by nucleotide sequencing (DNA Sequencing Laboratory, ICBR, University of Florida). The mutants contained only one amino acid mutation (cysteine to alanine) at either position Cys-976, Cys-991, Cys-1048, or Cys-1114.

Transient expression of wild-type ecNOS and mutants Cys-976, Cys-991, Cys-1048, or Cys-1114. COS-7 cells grown on 100-mm culture plates (~50% confluent) were transfected with antisense, wild-type, or mutant (Cys-976, Cys-991, Cys-1048, or Cys-1114) cDNAs using the Transfectam reagent (Promega) as described by the manufacturer.

The cells were incubated in serum-free RPMI 1640 medium containing 15 μg of Transfectam and 5 μg of DNA at 37°C for 5 hr. After incubation, cells were washed and reincubated in RPMI 1640 medium containing 10% fetal bovine serum at 37°C for 18-24 hr. The cells were harvested and used for characterization of ecNOS activity, for Western analysis, and for measurements of NADPH oxidation and cytochrome c reductase activity.

Measurement of ecNOS activity. Total cell membranes and cytosol fractions were isolated from antisense, wild-type, and mutant (Cys-976, Cys-991, Cys-1048, and Cys-1114) transfected cells by differential centrifugation as previously described (9,10). Total membrane and cytosol fraction ecNOS activities were measured by monitoring the formation of [3H] L-citrulline from [3H] L-arginine (11). Total membrane or cytosol fractions (100-120 μg protein each) were incubated (total volume 0.4 ml) in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM EDTA and EGTA, 1 mM PMSF, 10 mg/liter of leupeptin, 2.5 mM CaCl2, 1 mM NADPH, 10 μM tetrahydrobiopterin, 100 nM calmodulin, and 5 μM combined L-arginine and purified [3H] L-arginine (0.6 μCi; specific activity 69 Ci/mmol; NEN, Boston, MA) at 37°C for 30 min. Blanks were incubated under identical conditions in the absence of membrane or cytosolic proteins. In some experiments, ecNOS activity was measured in the presence of varying concentrations (50 to 1000 μM) of NADPH. In other experiments, ecNOS activity was measured in the presence of 50 μM N-nitro L-arginine methyl ester (L-NAME), an inhibitor of NOS. Purification of [3H] L-arginine and measurement of [3H] l-citrulline formation were carried out as previously described (9). The specific activity of ecNOS was determined by substracting the activity in the blank and is expressed as pmol L-citrulline/min/mg protein. The protein contents of total membrane and cytosol fractions were determined by the method of Lowry et al (12).

NADPH cytochrome c reductase and NADPH oxidation assays. NOS-catalyzed cytochrome c reductase and NADPH oxidation were measured as previously described (13). In brief, cytochrome c reductase activity was measured in a final volume of 0.5 ml Tris-HCl buffer (50 mM, pH 7.4) containing 100 μM free calcium, 100 nM calmodulin, 500 μM NADPH, 250 μM cytochrome c, and 100 μg membrane protein at 37°C. NADPH oxidation was measured in a final volume of 0.5 ml Tris-HCl buffer (50 mM, pH 7.4) containing 500 μM free calcium, 100 nM calmodulin, 500 μM NADPH, 10 μM tetrahydrobiopterin, 5 μM L-arginine, and 100 μg of membrane protein at 37°C. The changes in absorbance at 550 nm (cytochrome c) and 340 nm (NADPH) were continuously monitored against enzyme-deficient blank samples. Rates of cytochrome c reduction and NADPH oxidation were calculated using extinction coefficients of E550 = 21 mM-1 $\times$ cm-1 and E340 = 6.34 mM-1 $\times$ cm-1, respectively (13).

Protein extraction and Western analysis. Antisense, wild-type, and mutant (Cys-976, Cys-991, Cys-1048, and Cys-1114) transfected cells were washed twice with phosphate-buffered saline and then lysed in boiled SDS-PAGE sample buffer (0.06 M Tris-HCl, 2% SDS, and 5% glycerol, pH 6.8). The lysates were boiled in a water bath for 5 min to remove insoluble materials. The cell lysate proteins (20 μg) were fractionated on a 7.5% SDS-PAGE gel and blotted onto a polyvinylidene difluoride (PVDF, Bio-Rad) membrane as described by Burnette (14). The blot was incubated in blocking solution (3% BSA in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Tween 20 (TBST)) and then hybridized with anti-ecNOS monoclonal antibody (Transduction Laboratories, Lexington, KY, 1:5000 diluted in TBST) at room temperature for 1 hr. After washing, the membrane was incubated in 1:10,000 diluted anti-IgG horseradish peroxidase linked whole antibody (Amersham, Life Sciences, Arlington Heights, IL) for 1 hr. The immunoreactive bands were visualized by ECL (enhanced chemiluminescence) reagents (Amersham) with Kodak X-OMAT film. The protein contents of cell lysates were determined by the method of Lowry et al (12).

Statistical analysis. Statistical significance of differences in ecNOS activity between wild-type and mutants was determined by analysis of variance and Student's t-test (15).

RESULTS

Expression and characterization of ecNOS activity. To examine the contribution of reductase domain cysteinyl residues to the catalytic activity of ecNOS, the antisense, wild-type, and mutant (Cys-976, Cys-991, Cys-1048, and Cys-1114) cDNAs of human ecNOS were expressed in COS-7 cells, and the distribution of catalytic activity in total membrane and cytosol fractions, the effect of L-NAME on the catalytic activity, and ecNOS protein expression by Western analysis were examined. As shown in Fig. 1, significantly ($p < 0.01$) enhanced catalytic activities were observed in the total membrane fractions derived from wild-type- and Cys-976- and Cys-991-transfected cells compared to total membrane fractions derived from Cys-1048-, Cys-1114-, and antisense-transfected cells. Addition of L-NAME to the incubation mixture diminished the catalytic activity of ecNOS by more than 90% in wild-type as well as mutant- and antisense-transfected cells. ecNOS activities in cytosol fractions were less than 5% of the activity in the total membrane fractions of the wild-type and the mutants (not shown). In contrast to the activity measurements, Western analysis indicated that the expression of ecNOS protein, with a molecular mass of 135 kDa, was comparable in wild-type- and all

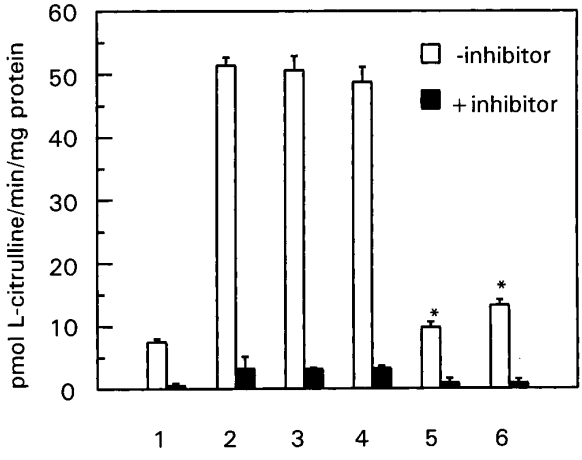

FIG. 1. Catalytic activity of ecNOS in antisense, wild-type, and mutants expressed in COS-7 cells. COS-7 cells were transfected with antisense cDNA (negative control), sense cDNA (wild-type), or Cys-976-, Cys-991-, Cys-1048-, and Cys-1114-mutated cDNAs as described in Methods. After a 24-hr incubation at 37°C, cells were washed, total membrane fractions were isolated, and NOS activity was measured by monitoring the formation of [3H]-citrulline from [3H]-L-arginine with and without the presence of 50 μM L-NAME (inhibitor). 1: native NOS activity in COS-7 cells; 2: wild-type; 3–6: mutation of cysteine residues at positions 976, 991, 1048, and 1114, respectively. Data represent means ± SE (n = 10) for each group. * $p < 0.01$ versus wild-type.

mutant-transfected cells (Fig. 2). Antisense-transfected cells did not show any band due to the low concentration of native NOS protein in COS-7 cells.

Effect of varying concentrations of NADPH on ecNOS activity. Mutations of Cys-1048 and Cys-1114 resulted in significant loss of catalytic activity of ecNOS. Because these mutants are located in the NADPH binding site of the reductase domain of ecNOS, we examined the potential effect of varying concentrations of NADPH on L-arginine metabolism by ecNOS wild type and mutants. As shown in Fig. 3, ecNOS activity in wild-type was NADPH concentration-

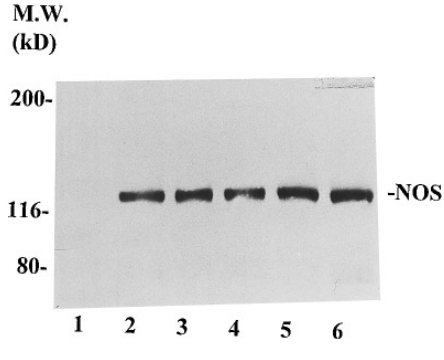

FIG. 2. Western blot analysis of antisense, wild-type, and mutated ecNOS expressed in COS-7 cells. COS-7 cells were transfected with antisense cDNA (negative control), sense cDNA (wild-type), or Cys-976-, Cys-991-, Cys-1048-, and Cys-1114-mutated cDNAs as described in Methods. After a 24-hr incubation at 37°C, cells were washed and lysed in boiled SDS-PAGE buffer (0.06 M Tris-HCl, 2% SDS, and 5% glycerol, pH 6.8). The cell lysate proteins (20 μg) were fractionated on SDS-PAGE, blotted on PVDF membranes, and hybridized with ecNOS monoclonal antibody as described in Methods. Lane 1, antisense; lane 2, wild-type; lane 3, Cys-976; lane 4, Cys-991; lane 5, Cys-1048; lane 6, Cys-1114. Cells transfected with antisense ecNOS cDNA did not show any band. Results are representative of two separate experiments with similar observations.

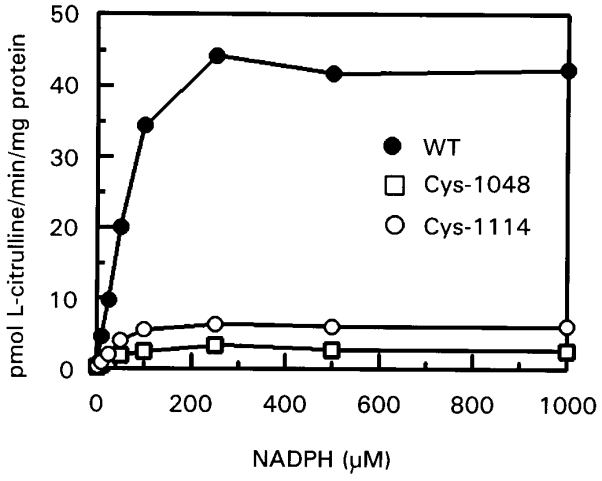

FIG. 3. Effect of varying concentrations of NADPH on ecNOS activity. Total membranes were isolated from wild-type (WT) and Cys-1048 and Cys-1114 mutant-transfected COS-7 cells. NOS activity was measured by incubating 100 μg of total membrane fraction protein in the presence of arginine, all cofactors, and varying concentrations (50 to 1000 μM) of NADPH at 37°C for 30 min as described in Methods. Data represent the mean of two separate experiments.

dependent. Maximal activity in the wild type was 45 pmol L-citrulline/min/mg protein and was achieved in the presence of 250 μM NADPH. However, in mutants cys-1048 and cys-1114, ecNOS activity was relatively constant irrespective of the NADPH concentration. The maximal ecNOS activity in the mutants was approximately 15% of the maximal activity in wild-type.

Effect of Cys-1048 and Cys-1114 mutations on NADPH oxidation. To confirm that mutations Cys-1048 and Cys-1114 affect utilization of NADPH during L-arginine metabolism, we monitored the rate of NADPH oxidation by wild-type and mutant ecNOS. As shown in Fig. 4, NADPH oxidation in the wild-type increased in a time-dependent fashion. After a 30-min incubation, NADPH oxidation was increased nearly 7-fold over that measured after a 5-min incubation. In contrast, NADPH oxidation increased less than 3-fold during the 30-min incubation period in mutants Cys-1048 and Cys-1114. The maximal utilization of NADPH was 5- to 7-fold higher in the wild-type than in mutants Cys-1048 and Cys-1114.

Effect of Cys-1048 and Cys-1114 mutations on cytochrome c reduction. To confirm that mutations Cys-1048 and Cys-1114 alter electron transfer function of the reductase domain of ecNOS, we determined the rate of cytochrome c reduction which is dependent upon the transfer of electrons from NADPH. As shown in Fig. 5, cytochrome c reduction was rapid and increased in a time-dependent fashion in the wild-type but not in mutants Cys-1048 and Cys-1114. The maximal reduction of cytochrome c was 4-fold greater in wild-type than in either mutant.

DISCUSSION

We have recently reported that thiol modulating chemicals reduce ecNOS catalytic activity in intact pulmonary artery endothelial cells, as well as in membranes and purified enzyme isolated from these cells, suggesting that specific cysteinyl residues are critical for catalytic activity of this enzyme (9). The amino acid sequence alignment of NOS isoforms from diverse species reveals the presence of highly conserved cysteines at positions 976, 991, 1048, and 1114 in the FAD-NADPH binding site of all NOS isoforms with the following consensus sequence motifs: xGVCSx, xVPCFx, xVFGCRx, and xCGDVx (Table 1). The results of the

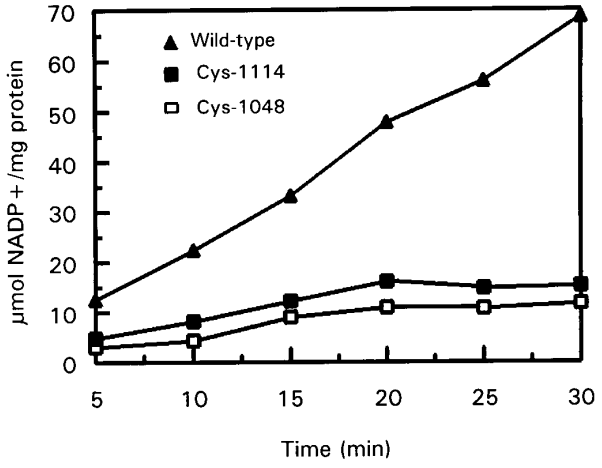

FIG. 4. Time-dependence of NADPH oxidation by wild-type and mutant ecNOS. Total membranes were isolated from wild-type and Cys-1048 and Cys-1114 mutant-transfected COS-7 cells. Total membrane fraction protein (100 μg) was incubated in Tris-HCl buffer containing 500 μM free calcium, 100 nM calmodulin, 500 μM NADPH, 10 μM tetrahydrobiopterin, and 5 μM L-arginine at 37°C. Oxidation of NADPH was monitored for 30 min at 340 nm as described in Methods. Data represent the mean of two separate experiments.

present study using oligonucleotide-directed mutation of Cys-976, Cys-991, Cys-1048, and Cys-1114 residues demonstrate for the first time that Cys-1048 and Cys-1114, but not Cys-976 and Cys-991, residues located in the reductase domain play a critical role in mediating the catalytic activity of human ecNOS.

The molecular structure of all NOS isozymes contains oxygenase and reductase domains linked by a calcium/calmodulin binding region (5,6). The C-terminal domain of the NOS protein shares sequence homology with the mammalian enzyme NADPH cytochrome P-450

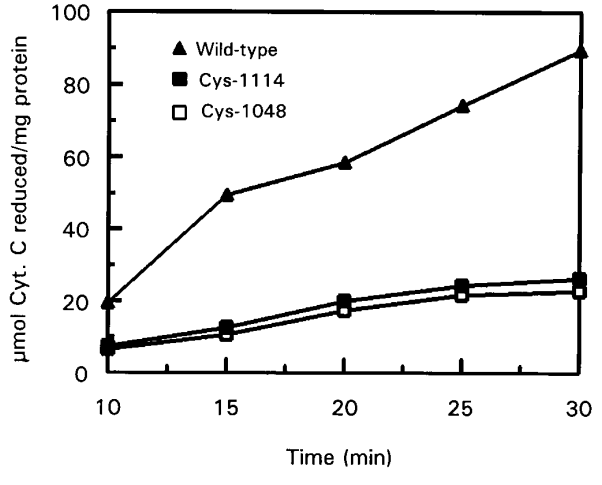

FIG. 5. Time-dependent reduction of cytochrome c by wild-type and mutant ecNOS. Total membranes were isolated from wild-type and Cys-1048 and Cys-1114 mutant-transfected COS-7 cells. Rate of cytochrome c reduction was monitored at 550 nm by incubating total membrane fraction protein (100 μg) in the presence of 100 μM free calcium, 100 nM calmodulin, 500 μM NADPH, and 250 μM cytochrome c in Tris-HCl buffer at 37°C for 30 min as described in Methods. Data represent the mean of two separate experiments.

reductase (4,16). This domain, which binds NADPH, FAD, and flavin mononucleotide (FMN), serves as the site of electron transfer to the heme in the oxygenase domain of NOS. On the basis of reported studies with NOS and other cytochrome P-450 enzymes, the probable flow of electrons occurs from NADPH to FAD-FMN to the heme site, resulting in the activation of molecular oxygen, subsequent oxidation of L-arginine, and formation of NO and L-citrulline (5,6). The mutation of Cys-976, Cys-991, Cys-1048, and Cys-1114 to alanine residues was designed to generate FAD-NADPH binding site mutants that would be likely to reduce the utilization of NADPH and/or electron transfer and therefore catalytic activity of ecNOS. Catalytic activity of the enzyme from the Cys-1048 and Cys-1114 mutants was several fold less than the activity in wild-type ecNOS or in Cys-976 and Cys-991 ecNOS mutants despite the fact that expression of ecNOS protein mass was comparable in wild-type and all mutants. Cys-1048 is in close proximity to the NADPH binding site, and Cys-1114 lies within the NADPH binding site. In contrast, Cys-976 and Cys-991 are close to the FAD binding site, suggesting that Cys-1048 and Cys-1114 residues are critical to the function of the NADPH binding site of human ecNOS.

The precise role of Cys-1048 and Cys-1114 in mediating ecNOS catalytic activity is not known but is likely to be associated with the binding of NADPH to the ecNOS protein or with electron transfer from NADPH to FAD. The impairments in NADPH oxidation and cytochrome c reduction in the Cys-1048 and Cys-1114 mutants suggest that these mutants are limited in their ability to utilize NADPH. This limitation in utilization of NADPH by mutant enzymes is consistent with a failure to bind NADPH to its active site due to a conformational change in protein structure or to a change in protein structure that interferes with electron transfer despite the binding of NADPH. These possibilities are consistent with the results of studies conducted in several reductase enzymes that require NADPH or NADH as an electron donor. For example, the binding characteristics of NADPH were reported to be influenced by conformational changes of glutathione reductase from human erythrocytes (17). Hackett et al (18) identified that the Cys-283 residue of bovine NADH-cytochrome b5 reductase was directly involved with enzyme activity. Similarly, Aliverti et al (19) reported that mutation of Cys-132 and Cys-272 residues of spinach flavoprotein ferredoxin-NADPH reductase resulted in a 7-fold decrease in its catalytic efficiency. It has been proposed that Cys-272 of ferredoxin-NADPH reductase plays a critical role in stabilizing the charge-transfer complex (FAD.-NADPH) between the reductase and NADPH and in facilitating electron transfer (19). Since cysteine residues are highly conserved in the NADPH binding site of the enzymes belonging to the NOS and ferredoxin-NADPH reductase families, Cys-1114 of human ecNOS may be assumed to have an analogous role in facilitating electron transfer and catalysis of L-arginine. Similarly, Cys-1048 may be critical in shuttling electrons from NADPH to FAD in ecNOS. Our ongoing studies are focused on identifying the precise role of these cysteinyl residues in electron transfer from the reductase to the oxygenase domain of human ecNOS.

Thiol modulation of ecNOS protein has several important physiologic implications. For example, maintaining normal vascular tone is dependent on NO production and therefore the catalytic activity of ecNOS. This is particularly relevant to the lung where oxidant-induced injuries resulting from exposure to environmental pollutants and thiol-reactive chemicals such as ozone and nitrogen dioxide, from herapeutic interventions such as supplemental oxygen administration, radiation, and chemotherapy, and from the use of authentic NO gas in inhalation therapy are common. The resultant oxidant stress from these conditions is likely to cause thiol modulation of proteins, including NOS.

ACKNOWLEDGMENTS

The authors thank Miss Rachel Derin for tissue culture assistance and Mrs. Di-hua He for technical assistance. We also thank Ms. Janet Wootten for excellent editorial help and Ms. Anne Hines for secretarial assistance. This work

was supported in part by the Medical Research Service of the Department of Veterans Affairs and by NIH Grant ES06219.

REFERENCES

1. Ignarro, L. J., Bugg, G. M., and Wood, K. S. (1987) *Circ. Res.* **61,** 866–879.
2. Palmer, M. J., and Moncada, S. (1989) **158,** 348–352.
3. Forstermann, U., Pollock, J. S., Schmidt, H. H. H. W., Heller, M., and Murad, F. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 1788–1792.
4. Sessa, W. C. (1994) *J. Vasc. Res.* **31,** 131–143.
5. Marletta, M. A. (1993) *J. Biol. Chem.* **268,** 12231–12234.
6. Kerwin, J. F., Jr., Lancaster, J. R., Jr., and Feldman, P. L. (1995) *J. Med. Chem.* **38,** 4343–4362.
7. Chen, P. F., Tsai, A. L., and Wu, K. K. (1994) *J. Biol. Chem.* **269,** 25062–25066.
8. Chen, P. F., Tsai, A. L., and Wu, K. K. (1995) *Biochem. Biophys. Res. Commun.* **215,** 1119–1129.
9. Patel, J. M., and Block, E. R. (1995) *Am. J. Respir. Cell Mol. Biol.* **13,** 352–359.
10. Patel, J. M., Zhang, J. L., and Block, E. R. (1996) *Am. J. Respir. Cell Mol. Biol.,* in press.
11. Bredt, D. S., and Snyder, S. H. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 682–685.
12. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
13. Klatt, P., Schmidt, K., Uray, G., and Mayer, B. (1993) *J. Biol. Chem.* **268,** 14781–14787.
14. Burnette, W. N. (1981) *Anal. Biochem.* **112,** 195–203.
15. Winer, B. J. (1971) Statistical Principles in Experimental Design, pp. 210–219, McGraw–Hill, New York.
16. Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H. (1991) *Nature* **351,** 714–718.
17. Pai, E. F. (1988) *Biochemistry* **27,** 4465–4474.
18. Hackett, C. S., Novoa, W. B., Ozols, J., and Strittmatter, P. (1986) *J. Biol. Chem.* **261,** 9854–9857.
19. Aliverti, A., Piubelli, L., Zanetti, G., Lubberstedt, T., Hermann, R. G., and Curti, B. (1993) *Biochemistry* **32,** 6374–6380.
20. Janssens, S. P., Simouchi, A., Quertermous, T., Bloch, D. B., and Bloch, K. D. (1992) *J. Biol. Chem.* **267,** 14519–14522.
21. Lamas, S., Marsden, P. A., Li, G. K., Tempst, P., and Michel, T. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6348–6352.
22. Geller, D. A., Lowenstein, C. J., Shapiro, R. A., Nussler, A. K., Di Silvio, M., Wand, S. C., Nakayama, D. K., Simmons, R. L., Snyder, S. H., and Billiar, T. R. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3491–3495.
23. Xie, Q. W., Cho, H. J., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T., and Nathan, C. (1992) *Science* **256,** 225–228.
24. Nunokaway, Y., Ishida, N., and Tanaka, S. (1993) *Biochem. Biophys. Res. Commun.* **191,** 89–94.
25. Nakane, M., Schmidt, H. H. H. W., Pollock, J. S., Forstermann, U., and Murad, F. (1993) *FEBS Lett.* **316,** 175–180.