

Alteration of the Heme Prosthetic Group of Neuronal Nitric-Oxide Synthase during Inactivation by N^G -Amino-L-arginine in Vitro and in Vivo

JENNIFER L. VULETICH, EZRA R. LOWE, SUREE JIANMONGKOL, YASUHIKO KAMADA, UTE M. KENT, ANDREW T. BENDER, DAMON R. DEMADY, PAUL F. HOLLENBERG, and YOICHI OSAWA

Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, Michigan

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ABSTRACT

It is established that N^G -amino-L-arginine (NAA) is a metabolism-based inactivator of all three major nitric-oxide synthase (NOS) isoforms. The mechanism by which this inactivation occurs, however, is not well understood. In the current study, we discovered that inactivation of the neuronal isoform of NOS (nNOS) by NAA in vitro results in covalent alteration of the heme prosthetic group, in part, to products that contain an intact porphyrin ring and are either dissociable from or irreversibly bound to the protein. The alteration of the heme is concomitant with the loss of nNOS activity. Studies with nNOS containing a ^{14}C -labeled prosthetic heme moiety indicate that the major dissociable product and the irreversibly bound heme adduct

account for 21 and 28%, respectively, of the heme that is altered. Mass spectral analysis of the major dissociable product gave a molecular ion of m/z 775.3 that is consistent with the mass of an adduct of heme and NAA minus a hydrazine group. Peptide mapping of the irreversibly bound heme adduct indicates that the heme is bound to a residue in the oxygenase domain of nNOS. We show for the first time that metabolism-based inactivation of nNOS occurs in vivo as highly similar heme products are formed. Because inactivation and alteration may trigger ubiquitination and proteasomal degradation of nNOS, NAA may be a useful biochemical tool for the study of these basic regulatory processes.

Nitric-oxide synthases (NOSs) are P450-like hemoprotein enzymes that catalyze the NADPH-dependent oxidation of L-arginine to citrulline and NO (McMillan et al., 1992; Stuehr and Ikeda-Saito, 1992; White and Marletta, 1992). These enzymes are bi-domain in structure with an amino-terminal oxygenase domain, which contains binding sites for the heme prosthetic group and (6*R*)-5,6,7,8-tetrahydro-L-biopterin, and a carboxyl-terminal reductase domain, containing binding sites for FMN, FAD, and NADPH (Roman et al., 1995). These enzymes must also bind Ca^{2+} /calmodulin, at a site between the two domains, to elicit maximal NO-forming activity. For the neuronal and endothelial isoforms, binding of calmodulin and activation of the enzyme occurs in response to elevations in cytosolic calcium levels. In contrast, the inducible isoform of NOS binds Ca^{2+} /calmodulin with high affinity and is not

regulated by physiological levels of calcium (Stuehr et al., 1991).

NO signaling plays a vital role in a variety of physiological processes, such as neurotransmission, vasorelaxation, platelet aggregation, and immune response; yet, it is also involved in many pathological conditions including septic shock, reperfusion injury, arthritis, and atherosclerosis. Consequently, the intracellular mechanisms that regulate NOS expression and activation are of great importance. Our laboratory has focused on the effects of guanidine drugs on the neuronal isoform of NOS (nNOS), with emphasis on the toxicological repercussions associated with some NOS inhibitors (Nakatsuka et al., 1998). In particular, we have been investigating the mechanism(s) that are responsible for the enhanced proteolytic degradation of inactivated nNOS (Noguchi et al., 2000).

The compound N^G -amino-L-arginine (NAA), which is an arginine analog that contains a hydrazine group on the guanidino moiety, has been described previously as a metabolism-based inactivator of all three NOS isoforms in vitro (Wolff and Lubeskie, 1996). Based on the reported K_i values,

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ABBREVIATIONS: NOS, nitric-oxide synthase; NO, nitric oxide; HEK, human embryonic kidney; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; nNOS, neuronal NOS; nNOS(^{14}C heme), apo-nNOS reconstituted with ^{14}C -labeled heme; NAA, N^G -amino-L-arginine; ECL, enhanced chemiluminescence; ESI-LC-MS, electrospray ionization-liquid chromatography-mass spectrometry; DTT, dithiothreitol; A23187, calcium ionophore A23187.

however, nNOS ($K_i = 0.3 \mu\text{M}$) does seem to be slightly more sensitive to inactivation by NAA compared with the endothelial ($K_i = 2.5 \mu\text{M}$) or inducible ($K_i = 3 \mu\text{M}$) isoforms of NOS (Wolff and Lubeskie, 1996). NAA has also been demonstrated to be highly effective against the NOS isoforms in vivo by antagonizing L-arginine-mediated endothelium-dependent relaxation with greater potency than N^G -methyl-L-arginine, another metabolism-based inactivator of NOS (Fukuto et al., 1990). Nonetheless, the in vivo use of NAA has been limited because of the propensity of this compound to cause neuromuscular rigidity and seizure-like activity in dogs (Cobb et al., 1992; Kilbourn et al., 1992). The exact mechanism by which nNOS is inactivated by NAA, and the relevance of this process to NAA-mediated toxicity, remains to be determined.

Bryk et al. (1999) recently demonstrated that treatment of nNOS with NAA in vitro causes a 50% loss in heme fluorescence and concluded that destruction of one heme per dimer is sufficient for complete inactivation of nNOS (Bryk and Wolff, 1999). These authors did not consider the possibility that alteration of the heme prosthetic group, as opposed to destruction, can lead to formation of heme products that retain, in part, their ability to fluoresce (Osawa et al., 1989, 1990; Jianmongkol et al., 2000). In the current study, we characterize two major altered heme products that are formed during inactivation of nNOS by NAA: a major dissociable product that seems to result from the reaction of a NAA metabolite with the heme prosthetic group and an irreversibly bound heme adduct of nNOS created by cross-linking of the heme to an amino acid(s) within the oxygenase domain of nNOS. Moreover, when the formation of altered heme products are taken into account, the extent of the decrease in heme is nearly equal to the extent of activity loss.

The metabolism-based inactivation of nNOS in vivo has not been demonstrated. We show for the first time that NAA causes the alteration of heme and loss of nNOS activity in intact HEK 293 cells by a mechanism highly similar to that defined in in vitro studies with the purified nNOS. This is significant in that previous studies (Cooper et al., 1998; Bryk and Wolff, 1999) indicated that nNOS is "refractory" to metabolism-based inactivation. Because metabolism-based inactivators enhance the turnover of nNOS in vivo (Noguchi et al., 2000), the current study indicates that NAA can be used as a tool to delineate the nature of the covalent alterations that render nNOS susceptible to degradation. Moreover, this study furthers our understanding of the processes involved in the inactivation of nNOS in vivo, which may be important in understanding the safety and efficacy of NOS inhibitors in general, as well as the mechanism of NAA-mediated toxicity, in particular.

Experimental Procedures

Materials. N^G -Amino-L-arginine hydrochloride was purchased from Alexis Biochemicals (San Diego, CA). Trypsin (tosylphenylalanyl chloromethyl ketone-treated, bovine pancreas), trypsin inhibitor (type 1S soybean), glucose 6-phosphate, glucose-6-phosphate dehydrogenase, calmodulin (crude, from bovine brain), horse heart myoglobin, NADP⁺, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). (6R)-5,6,7,8-Tetrahydro-L-biopterin was purchased from Dr. Schirck's Laboratory (Jona, Switzerland). Tris(2-carboxyethyl)phosphine and Super Signal West Pico enhanced chemiluminescence reagents were from Pierce (Rockford, IL). ¹⁴C-labeled heme (130 mCi/mmol) was purchased from the University of Leeds Indus-

trial Services (Leeds, UK). The affinity-purified rabbit IgG against brain NOS used for immunoblotting nNOS was from Transduction Laboratories (Lexington, KY).

Expression and Purification of nNOS. Rat neuronal NOS was expressed in Sf9 insect cells using a recombinant baculovirus and purified by 2',5'-ADP-Sepharose and gel filtration chromatography as described previously (Bender et al., 1999, 2000a). The nNOS([¹⁴C]heme) was prepared by in vitro reconstitution of apo-nNOS with ¹⁴C-labeled heme as described previously (Bender et al., 2000b) except that the reconstituted enzyme was resubmitted to ADP-Sepharose chromatography and concentrated on a Centricon concentrator (Millipore Corp., Bedford, MA). The rate of NO production catalyzed by the nNOS([¹⁴C]heme) was 640 nmol/min/mg of protein. The heme content was 0.42 mol of heme per mol of monomer.

Treatment of nNOS with NAA and NOS activity assay. Unless otherwise indicated, nNOS (0.5 μM) was added to a "first reaction mixture" of 50 mM potassium phosphate, pH 7.4, containing 0.2 mM CaCl₂, 100 U/ml superoxide dismutase, 25 U/ml catalase, 40 $\mu\text{g/ml}$ calmodulin, 10 μM (6R)-5,6,7,8-tetrahydro-L-biopterin, 0.4 mM NADP⁺, 10 mM glucose 6-phosphate, 1 unit/ml glucose-6-phosphate dehydrogenase, and 50 μM NAA in a total volume of 150 μl at room temperature. Aliquots (10 μl) of the first reaction mixture were transferred to an "oxyhemoglobin assay mixture" containing 200 μM CaCl₂, 100 μM NADPH, 100 μM L-arginine, 100 μM (6R)-5,6,7,8-tetrahydro-L-biopterin, 100 U/ml catalase, 10 $\mu\text{g/ml}$ calmodulin, and 25 μM oxyhemoglobin in a total volume of 200 μl of 50 mM potassium phosphate, pH 7.4. The mixture was incubated at 37°C and the rate of NO-mediated oxidation of oxyhemoglobin was monitored by measuring the absorbance at $\lambda_{401-411 \text{ nm}}$ with a microtiter plate reader (SpectraMax Plus; Molecular Devices, Menlo Park, CA) as described previously (Bender et al., 1999). Where indicated, the concentration of nNOS in the first reaction mixture was changed, and the amount of calmodulin was proportionally increased.

High Performance Liquid Chromatography (HPLC). HPLC was performed with the use of a Waters 600S controller, 717 Plus autosampler, and 996 photodiode array detector (Waters Corp., Milford, MA). Samples were injected onto a reverse phase HPLC column (5 μm , 0.21 \times 15 cm; C4 Vydac; Vydac, Hesperia, CA) equilibrated with solvent A (0.1% trifluoroacetic acid) at a flow rate of 0.3 ml/min. A linear gradient was run to 75% solvent B (0.1% trifluoroacetic acid in acetonitrile) over 30 min and then to 100% solvent B over the next 5 min. Absorbance at 220 and 400 nm was monitored. In experiments in which ¹⁴C-labeled heme was analyzed, the column was equilibrated with 25% solvent B at a flow rate of 0.3 ml/min. After 15 min of isocratic flow, a linear gradient to 75% and 100% solvent B was run over 20 min and 5 min, respectively. An on-line radiochemical detector (Radiomatic 500TR; Packard BioScience, Meriden, CT) was used to detect the radiolabeled products.

Electrospray LC-MS Analysis (ESI-LC-MS). ESI-LC-MS was accomplished using a Thermoquest LCQ LC-MS system (Thermo Finnigan, San Jose, CA), connected to a Hewlett Packard series 1100 binary pump and autosampler (Hewlett Packard Analytical Direct, Wilmington, DE). The LCQ was optimized for heme using myoglobin as a standard. The sheath gas and the auxiliary gas were set at 90 and 30 (arbitrary units), respectively. The spray voltage was 4.2 kV, and the capillary temperature was 200°C. HPLC conditions were the same as those described for samples containing ¹⁴C-labeled heme. After the first 5 min of flow, the LC effluent was infused directly into the LCQ. Peak 1 eluted from the LCQ at 8.2 min, followed by heme at 19.7 min, and nNOS protein at 22.4 min. For MS-MS analysis of peak 1, the solvent flow was decreased to 0.1 ml/min and the ion at m/z 775.3 was subjected to collision energy of 20%.

SDS-Polyacrylamide Gel Electrophoresis, Western Blotting, and ECL Detection of Protein-Bound Heme. The first reaction mixtures were added to an equal volume of sample buffer containing 5% SDS, 20% glycerol, 100 mM tris(2-carboxyethyl)phosphine, and 0.02% bromphenol blue in 125 mM Tris-HCl, pH 6.8. Samples were incubated for 10 min at 50°C before being loaded onto

gels, unless otherwise indicated. Samples were then subjected to electrophoresis on 7.5% SDS-polyacrylamide gels (10 × 8 cm) and transferred to nitrocellulose membranes (0.2 μm; Bio-Rad, Hercules, CA). The protein-associated heme was detected with the use of Super Signal West Pico enhanced chemiluminescence detection reagents (Pierce) and X-Omat film (Eastman Kodak, Rochester, NY) as described previously (Vuletich and Osawa, 1998). nNOS protein was visualized by Western blotting using 0.01% anti-nNOS polyclonal antibody from Transduction Laboratories. An anti-rabbit IgG conjugated to peroxidase (Roche Applied Science, Indianapolis, IN) was used as secondary antibody at a concentration of 0.01%.

Trypsinolysis of nNOS. For analysis by SDS-PAGE, nNOS (5 μM) was treated with NAA in the first reaction mixture as described above. An aliquot (20 μl) of the reaction mixture was added to 30 μl of 50 mM Tris-HCl, pH 7.6, containing 1 mM DTT, 20 mM EDTA, and *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (2,000 mU/ml). At the given time points, aliquots (10 μl) were removed and quenched with an equal volume of gel sample buffer containing soybean trypsin inhibitor (15 μg/ml). The entire sample (20 μl) was then analyzed by SDS-PAGE as described above, except that 10% gels were used. For HPLC analysis of tryptic heme peptides, nNOS (1.5 μM) was treated with NAA (500 μM) for 60 min as described above. The reaction mixture (180 μl) was submitted to the HPLC procedure described above, and the fraction corresponding to the heme irreversibly bound to the protein was collected. The sample was dried to completeness with the use of a SpeedVac (Thermo Savant, Holbrook, NY) and subsequently dissolved in 300 μl of 50 mM Tris-HCl, pH 7.6, containing 1 mM DTT. *N*-Tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (10,880 mU/ml as a final concentration) was added, and the mixture was incubated at 37°C. Aliquots (135 μl) were taken for analysis by HPLC as described above.

Cell Culture and Preparation of the Cytosolic Fraction. Human embryonic kidney (HEK) 293 cells stably transfected with rat nNOS by Bredt et al. (1991) were obtained from Dr. Bettie Sue Masters (University of Texas Health Science Center, San Antonio, TX). HEK 293 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% calf serum (Hyclone), 20 mM HEPES, pH 7.4, and G418 (0.5 mg/ml; Geneticin; Invitrogen) as described previously (McMillan et al., 1992). Before each experiment, the cells were cultured in DMEM containing 0.1 mM arginine (low-arginine DMEM) for at least 12 h. HEK cells were harvested in their treatment medium diluted 1:1 with ice-cold phosphate-buffered saline. The cells were then pelleted, washed once with 10 ml of ice-cold phosphate-buffered saline, and pelleted again. The cell pellet was homogenized on ice with a Tenbroeck ground glass homogenizer (Kimble/Kontes, Vineland, NJ) in buffer containing 10 mM HEPES, pH 7.4, 0.32 M sucrose, 0.1 mM EDTA, 1.5 mM DTT, 10 μg/ml trypsin inhibitor, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mg/ml phenylmethylsulfonyl fluoride. Homogenates were centrifuged for 10 min at 16,000g, the supernatant was removed, and homogenates were centrifuged for an additional 15 min at 100,000g to obtain a cytosolic fraction.

Data Analysis. Statistical differences between treatment groups were evaluated using one-way analysis of variance followed by a post hoc Dunnett's test. A *p* value less than 0.01 was considered statistically significant.

Results

Effect of NAA on nNOS Activity. As shown in Fig. 1, treatment of recombinant nNOS with 50 μM NAA resulted in a time-dependent decrease in nNOS activity, which was measured by the oxyhemoglobin assay (Fig. 1, ▲). Moreover, this activity loss was dependent upon the presence of calmodulin (Fig. 1, compare ▲ with ●), which is necessary for nNOS activity, and indicates a metabolism-dependent inactivation

process. The slight decrease in activity observed when nNOS was treated with calmodulin alone (Fig. 1, ■) illustrates the propensity of this enzyme to autoinactivate in the absence of substrate. The findings for NAA are consistent with a suicide mechanism of inactivation whereby a substrate is metabolized to a reactive intermediate that covalently alters nNOS and inactivates the enzyme. These results are also consistent with those established for the NAA-mediated inactivation of nNOS purified from GH₃ pituitary cells (Wolff and Lubeskie, 1996). Because the kinetics of this reaction were reported previously (Wolff and Lubeskie, 1996), we chose to focus on the mechanism of inactivation of nNOS by NAA.

Alteration of the Heme Prosthetic Group of nNOS by NAA. The HPLC profile for the reaction mixture containing nNOS treated with 50 μM NAA in the absence of calmodulin is shown in Fig. 2A. The major peak with absorption at 400 nm (Fig. 2A, solid line) corresponds to native heme (Fig. 2A, Heme), which dissociates from the protein under the acidic conditions of the chromatography, and the major peak with absorption at 220 nm (Fig. 2A, dashed line) corresponds to the nNOS apoprotein. As shown in Fig. 2B, treatment of nNOS with 50 μM NAA in the presence of calmodulin resulted in a loss in the peak area for native heme and the formation of two new peaks with absorption at 400 nm (Fig. 2B, peaks 1 and 2). The chromatogram at 220 nm shows the nNOS apoprotein peak at approximately 26.5 min, as well as several other peaks at 22, 24.5, and 28 min, that were due to other components that were present in the calmodulin preparation. In the chromatogram at 400 nm, peak 1 corresponds to a dissociable heme product, and peak 2, which coelutes with the nNOS apoprotein, corresponds to an altered heme product that is irreversibly bound to the protein. The spectrum of the fraction that corresponds to heme, peak 1, or peak 2 was determined by on-line diode array analysis (Fig. 2B, inset). All three samples were observed to have the char-

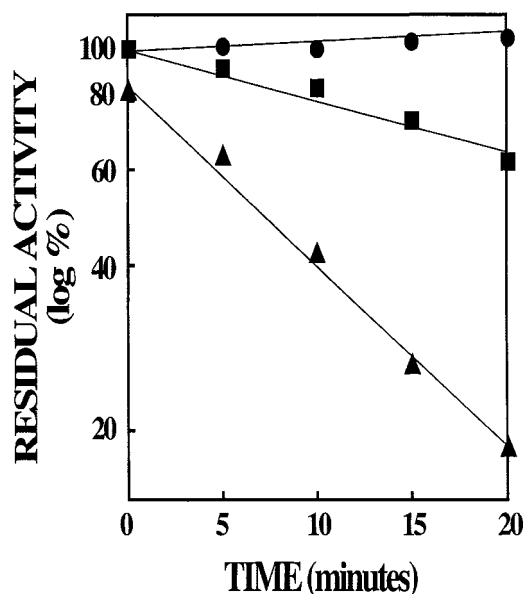


Fig. 1. Time- and calmodulin-dependent inactivation of nNOS by NAA. The time-dependent inactivation of nNOS was determined with the use of the first reaction mixture and the oxyhemoglobin assay as described under *Experimental Procedures*. ●, nNOS treated with 50 μM NAA in the absence of calmodulin; ▲, nNOS treated with 50 μM NAA in the presence of calmodulin; ■, nNOS treated with calmodulin alone.

acteristic Soret absorbance of porphyrin compounds. The Soret maximum for peak 1 was found to be 405 nm, which is slightly red-shifted compared with the Soret for native heme at 398 nm. The Soret maximum for peak 2 was 398 nm, which was similar to heme. Because of potential differences in absorptivity of the altered heme products and the lack of standards, they could not be accurately quantified by their absorbance.

Quantitation of the NAA-Mediated Alteration of Heme and the Formation of Altered Heme Products. As shown in Fig. 3, unlike the altered heme products, the amount of heme observed in the HPLC profile at 400 nm can be quantified and compared with the loss in nNOS activity under the same conditions. The decreases in heme and nNOS activity were both found to be time-dependent (Fig. 3A). The decrease in heme accounted for 84% of the activity loss (Fig. 3B, condition 1). As shown in Fig. 3B, these changes in heme and activity only occurred when NAA was incubated with nNOS in the presence of calmodulin (Fig. 3B; compare condition 1 with condition 2), indicating a metabolism-dependent effect. The loss in activity and the decrease in heme were also dependent on NAA (Fig. 3B; compare condition 1 with condition 3). The decrease in heme, similar to the loss in nNOS activity, could also be attenuated by the natural substrate L-arginine (Fig. 3B, condition 4) but not by D-arginine (Fig. 3B, condition 5).

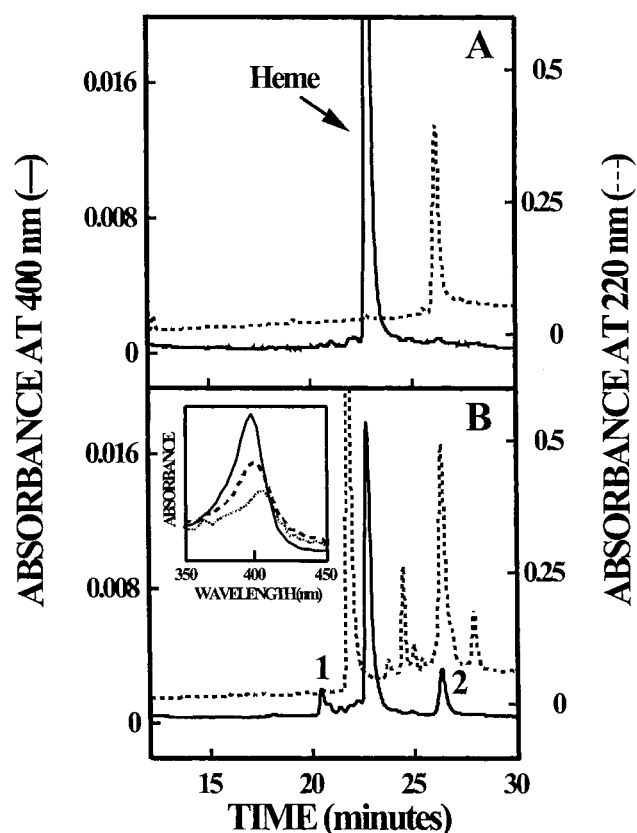


Fig. 2. HPLC profiles of nNOS treated with NAA in the absence (A) or presence (B) of calmodulin. nNOS (0.5 μ M) was treated with 50 μ M NAA for 20 min, and an aliquot (120 μ l) of each sample was analyzed by HPLC as described under *Experimental Procedures*. Inset, the absorption spectra of heme (solid line), peak 1 (dotted line), and peak 2 (dashed line) obtained by on-line diode array analysis of the HPLC effluent. The Soret maxima for heme, peak 1, and peak 2 were observed at 398, 405, and 398 nm, respectively.

The altered heme products, peak 1 and peak 2, were quantified using a preparation of apo-nNOS that was reconstituted with 14 C-labeled heme (nNOS(14 C]heme). The HPLC method used in these experiments was modified from that described above to obtain better separation among peak 1, heme, and peak 2. The HPLC and radiochemical profiles of nNOS(14 C]heme) that was incubated with 50 μ M NAA in the absence of calmodulin are shown in Fig. 4A. In both profiles, the peak corresponding to native heme elutes at approximately 23 min (Fig. 4A, Heme). The small peak eluting at approximately 31 min corresponds to heme that is irreversibly bound to the protein. Because this peak was also present in the starting material of nNOS(14 C]heme) (data not shown), it is most probably formed during the reconstitution process (Bender et al., 2000b). Incubation of nNOS(14 C]heme) with 50 μ M NAA in the presence of calmodulin was found to cause about a 50% decrease in the amount of native heme (Fig. 4B). Peak 1 and peak 2 accounted for approximately 21 and 28%, respectively, of the radioactive heme that was altered. The remainder of the radioactivity could be found in other dissociable heme products and in fractions that no longer absorb at 400 nm (fractions 2–20 min).

Characterization of the Dissociable Heme Product. The major dissociable heme product (*peak 1*) formed during inactivation of nNOS by NAA was further analyzed by ESI-MS. As shown in Fig. 4C, the mass spectrum of this compound gave a molecular ion multiplet with an ion of the highest intensity in this cluster at m/z 775.3. This mass is consistent with the addition of a molecule of NAA to the heme prosthetic group after the loss of the hydrazine moiety. Loss of the NAA portion from this adduct afforded the ion at m/z 616.4, which corresponds to heme. To further verify this relationship, we found that collision activated decomposition of the molecular ion at m/z 775.3 gave an ion at m/z 616.3, which corresponds to heme (Fig. 4D).

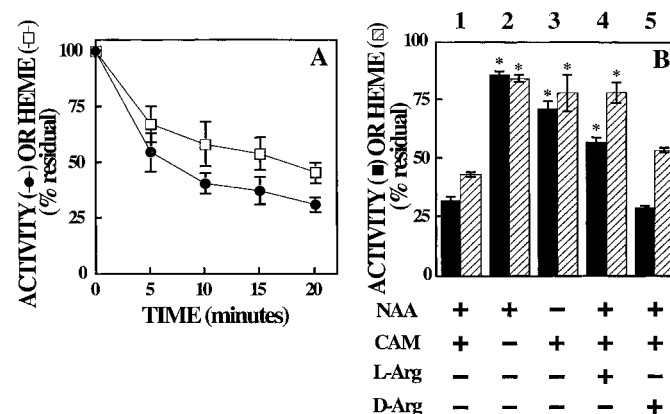


Fig. 3. Effect of NAA, calmodulin, and L- or D-arginine on nNOS activity and heme. A, nNOS (0.5 μ M) was incubated with 50 μ M NAA in the first reaction mixture, and aliquots (120 μ l) were removed immediately after mixing or after 5, 10, 15, and 20 min for measurement of heme (□) as described under *Experimental Procedures*. Aliquots (10 μ l) were taken at the same time for measurement of nNOS activity (●). B, the first reaction mixture containing nNOS was incubated for 20 min with NAA, calmodulin, 50 μ M L-arginine, or 50 μ M D-arginine as shown. The nNOS activity (solid bars) and the amount of heme (hatched bars) were determined as described under *Experimental Procedures*. The data represent the mean \pm S.E. from three separate experiments. *, significantly different from condition 1 ($p < 0.01$).

Characterization of the Irreversibly Bound Heme Adduct. The major heme product that remained associated with nNOS protein when analyzed by HPLC was further characterized by a recently developed method, involving SDS-PAGE and enhanced chemiluminescence (Vuletich and Osawa, 1998). The specificity of this assay is based on the ability of SDS-PAGE to separate heme that is irreversibly bound to protein from native heme that is dissociated from the protein and runs at the dye front (Vuletich and Osawa, 1998). As shown in Fig. 5, reaction mixtures containing nNOS were analyzed by the ECL assay (Fig. 5, top) under three separate conditions, and the monomer/dimer content of the samples under each condition was determined by Western blotting for nNOS protein (Fig. 5, bottom). In condition I, the nNOS remained associated as a SDS-resistant dimer (D) during electrophoresis (Fig. 5, bottom, lanes 1–3). In condition III, the samples were warmed slightly to promote dissociation of the nNOS into monomers (M) (Fig. 5, bottom, lanes 7–9). An intermediate condition, condition II, in which samples were warmed for a shortened duration, was also included (Fig. 5, bottom, lanes 4–6). As shown in the top of Fig. 5, condition I, we unexpectedly discovered that heme tightly associated with the nNOS dimer could be detected by the ECL assay (Fig. 5, lanes 1–3). However, nNOS that was treated with 100 μ M NAA in the presence of calmodulin also gave a chemiluminescence signal at a mass of approximately 160 kDa, which corresponds to heme that is irreversibly bound to the monomer of nNOS (Fig. 5, top, lane 2). Under condition III, most of the nNOS separated into monomers, and an intense chemiluminescence signal corresponding to irreversibly bound heme (Fig. 5, top, lane 8) was observed in the sample of nNOS that was inactivated with NAA. No signal was observed when nNOS was incubated with NAA in the absence of calmodulin (Fig. 5, top, lane 9). Interestingly,

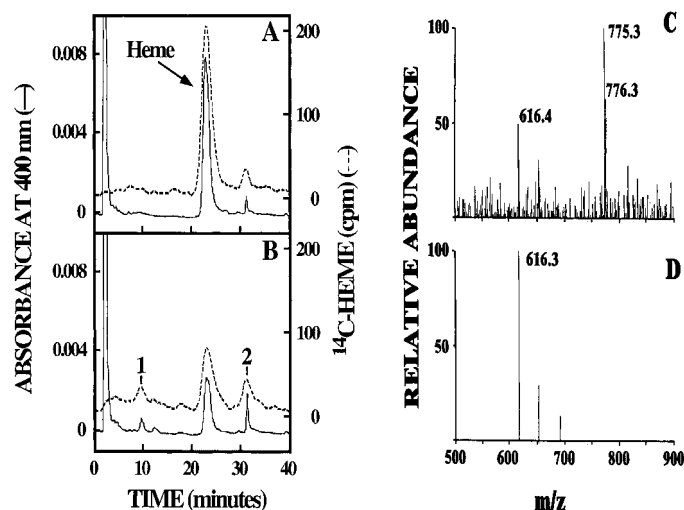


Fig. 4. HPLC profiles of nNOS, containing a ^{14}C -labeled heme group, treated with NAA and characterization of the major dissociable heme product. nNOS(^{14}C heme) (130 mCi/mmol) was incubated with 50 μ M NAA, with or without calmodulin, and aliquots (120 μ l) were analyzed by HPLC and on-line radiochemical detection as described under *Experimental Procedures*. A, nNOS treated with NAA in the absence of calmodulin. B, nNOS treated with NAA in the presence of calmodulin. C, nNOS (1 μ M) was incubated with 200 μ M NAA in the first reaction mixture for 60 min. An aliquot (50 μ l) of this sample was then analyzed by ESI-LC-MS. The compound corresponding to peak 1 in B was analyzed. D, MS-MS spectrum of the collision-activated fragmentation of the molecular ion at m/z 775.3.

a faint signal was also detected when nNOS was treated with calmodulin alone (Fig. 5, top, lane 7). Because no signal was observed for untreated nNOS (not shown), these results suggest that irreversibly bound heme adducts can also form during the autoinactivation process. Analysis of the samples under condition II revealed that nNOS inactivated with NAA or treated with calmodulin alone could more easily be dissociated into monomers than nNOS treated with NAA in the absence of calmodulin (Fig. 5, compare lanes 4 and 5 with lane 6).

To determine the site of heme attachment to nNOS, tryptic mapping of NAA-inactivated nNOS and analysis of the resulting peptides by the use of the ECL assay under condition III described above was attempted (Fig. 6). Trypsin is known to cleave nNOS preferentially at arginine residue 727, which is contained within the calmodulin binding region of the protein (Salerno et al., 1997). Cleavage at this site results in the formation of a 77-kDa fragment, corresponding to the carboxyl-terminal reductase domain of nNOS, and an 85-kDa fragment, which represents the amino-terminal oxygenase domain (Salerno et al., 1997). Figure 6A illustrates the time course of the formation of two major peptide bands at 77 and 85 kDa during limited trypsinolysis of NAA-inactivated nNOS. After digestion and analysis with the ECL assay, the chemiluminescence signal was primarily associated with the 85-kDa fragment (Fig. 6B). Thus, the irreversibly bound heme was contained within the oxygenase domain of nNOS.

Effect of NAA on nNOS in Transfected HEK 293 Cells. The HPLC profile of cytosol from HEK cells treated with the calcium ionophore, A23187, for 60 min is shown in Fig. 7A, top. A single peak in the 400-nm chromatogram corresponding to heme (Fig. 7A, Heme) was observed at approximately 23 min. The amount of heme in nNOS-transfected HEK cells was determined to be almost 5-fold greater than that in nontransfected HEK control cells and is thus mostly attributable to nNOS (data not shown). Treatment of

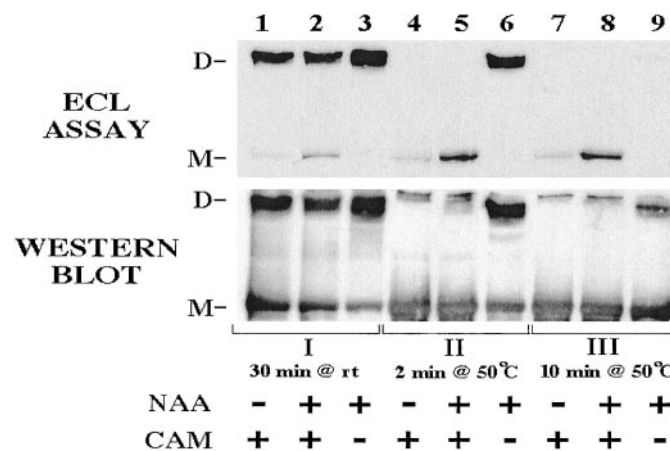


Fig. 5. ECL detection of heme irreversibly bound to the nNOS monomer or tightly associated with nNOS dimer. nNOS (1 μ M) was incubated with 100 μ M NAA for 60 min in the first reaction mixture, with (lanes 2, 5, and 8) or without (lanes 3, 6, and 9) calmodulin (CAM), as described under *Experimental Procedures*. A control with calmodulin but without NAA is also shown (lanes 1, 4, and 7). Before electrophoresis, aliquots (20 μ l) from the first reaction mixture were combined with an equal volume of sample buffer, incubated under the conditions indicated at the bottom of the figure, and subjected to SDS-PAGE on 7.5% gels as described under *Experimental Procedures*. Top, results of the ECL assay for heme; bottom, Western blot for nNOS protein. The positions of nNOS dimer (D) and monomer (M) are indicated on each blot.

HEK 293 cells with 100 μ M NAA for 60 min resulted in a decrease in heme, with the concomitant formation of two

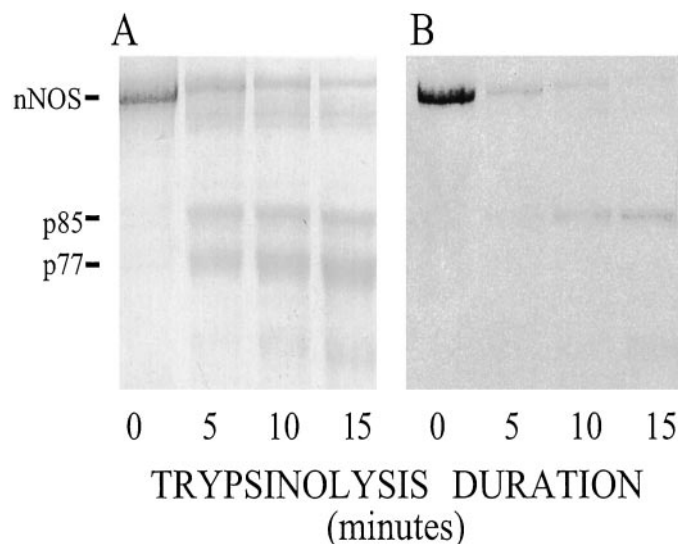


Fig. 6. Limited trypsinolysis of the nNOS-heme adduct. An aliquot (20 μ l) of the reaction mixture containing nNOS (5 μ M) treated with 100 μ M NAA for 60 min was transferred to a trypsinolysis reaction mixture and incubated for the indicated duration, as described under *Experimental Procedures*. Samples were prepared for electrophoresis and heated for 10 min at 50°C to form the nNOS monomer, as described under *Experimental Procedures*. A, Coomassie-stained gel; B, results of ECL assay for heme irreversibly bound to nNOS protein.

major altered heme products (Fig. 7A, bottom). The Soret maxima for peak 1 and peak 2 were 405 nm and 398 nm, respectively (data not shown), and were highly similar to those observed previously during inactivation of nNOS in vitro. With the use of the ECL assay, we also observed a signal for the protein bound heme adduct of nNOS in cells treated with NAA (Fig. 7A, bottom, inset; compare lane 2 with lane 1). Moreover, the formation of the protein-bound heme was attenuated by cotreatment of cells with 1 mM L-arginine (Fig. 7A, lane 3) but not by D-arginine (Fig. 7A, lane 4).

As shown in Fig. 7B, treatment of HEK cells with 100 μ M NAA in the presence of A23187 caused a time-dependent decrease in both nNOS activity and heme found in cytosol prepared from these cells. To calculate the amount of heme, the value obtained from nontransfected cells was subtracted from the value obtained in transfected cells. As shown in the inset (Fig. 7B), NAA caused a concentration-dependent decrease in nNOS activity of cytosol prepared from cells that were treated for 15 min. As shown in Fig. 7C, the activity loss and alteration of the heme only occurred when cells were incubated with NAA in the presence of ionophore (Fig. 7C, condition 1). Moreover, the alteration of heme accounted for approximately 82% of the activity that was lost under these conditions. Treatment with NAA or ionophore alone had little effect on either nNOS activity or heme in HEK 293 cells (Fig. 7C, conditions 2 and 3). Both the loss in nNOS activity and the heme alteration could be attenuated by 1 mM L-arginine

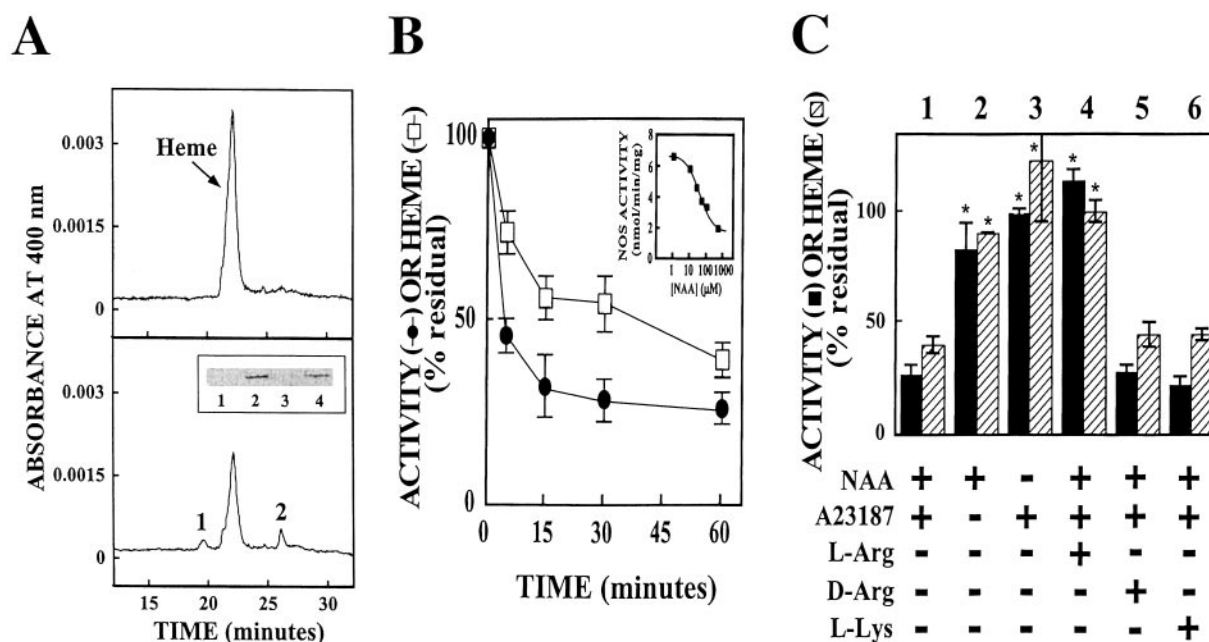


Fig. 7. Formation of altered heme products after treatment of HEK 293 cells with NAA. Samples of cell cytosol (100 μ g of protein) were analyzed by HPLC as described under *Experimental Procedures*. A, chromatogram of cytosol from HEK cells treated with 4 μ M A23187 for 60 min (top) and chromatogram of cytosol from HEK cells treated with 100 μ M NAA and 4 μ M A23187 for 60 min (bottom). The Soret maxima for heme, peak 1, and peak 2 were observed at 398, 405, and 398 nm, respectively. The bottom inset shows the ECL assay for heme irreversibly bound to nNOS protein found in 25 μ g of cytosolic protein prepared from HEK 293 cells as described under *Experimental Procedures*. Lane 1, cells treated for 60 min with medium containing 4 μ M A23187; lane 2, cells treated for 60 min with medium containing 100 μ M NAA and 4 μ M A23187; lane 3, same as in lane 2 except that 1 mM L-arginine was present; lane 4, same as in lane 2 except that 1 mM D-arginine was present. B, HEK 293 cells were treated with 100 μ M NAA and 4 μ M A23187 for the indicated duration. The cells were then harvested, cytosol was prepared, and aliquots (100 μ g of protein) were analyzed for heme (\square) by HPLC, and aliquots (20 μ l) were analyzed for nNOS activity (\bullet) as described under *Experimental Procedures*. Inset, dose-dependent inhibition of nNOS activity in HEK 293 cells after a 15-min treatment with NAA. C, HEK cells were treated for 60 min with medium containing 100 μ M NAA, 4 μ M A23187, 1 mM L- or D-arginine, or 1 mM L-lysine as indicated. The nNOS activity (solid bars) and the amount of heme (hatched bars) in the cell cytosol was determined. The value obtained for the amount of heme in nontransfected cells was subtracted from the value obtained for heme in transfected cells. The amount of heme in transfected cells was approximately 5-fold greater than that of nontransfected cells. The data represent the mean \pm S.E. from at least three separate experiments. *, significantly different from condition 1 ($p < 0.01$).

(Fig. 7C, condition 4), but not by 1 mM D-arginine (Fig. 7C, condition 5). The mechanism by which amino acids and some nNOS inhibitors enter cells has been shown previously to involve a cationic amino acid transport system (Schmidt et al., 1993; Forray et al., 1995). Therefore, it is possible that the protective effect of L-arginine, as well as the lack of protection by D-arginine, is due to competitive effects at the stereospecific transporter, rather than at the nNOS active site. To partially address this point, HEK cells were treated with NAA and ionophore in the presence of 1 mM L-lysine (Fig. 7C, condition 6), another amino acid that is transported by the same cationic amino acid transport system as arginine (Schmidt et al., 1993; Forray et al., 1995). Under these conditions, no protection against the NAA-mediated loss of nNOS activity or heme alteration in HEK cells was observed.

Discussion

In the current study, we characterized the nature and extent of the alteration of heme that occurs during the metabolism-based inactivation of nNOS because of NAA in vitro systems with the use of purified proteins. Furthermore, we used this knowledge to prove that metabolism-based inactivation by NAA occurs in vivo in a manner that is highly similar to that found in the in vitro studies.

Our studies with the use of purified proteins showed that the alteration of heme could account for approximately 84% of the activity loss. Moreover, the NAA-mediated heme alteration was time- and metabolism-dependent, and could be attenuated by the natural substrate L-arginine. These results were analogous to those observed for the NAA-mediated loss of enzymatic activity. Thus, the heme alteration and activity losses are intimately linked. We also discovered that the heme of nNOS is altered, in part, to a dissociable heme product and an irreversibly bound heme adduct. Together, these products accounted for almost half of the heme that was altered and thereby represent major pathways of heme modification. Bryk et al. (1999) have previously shown that NAA causes a loss in the fluorescence due to nNOS heme as measured in oxalic acid-derivatized, boiled samples of the reaction mixtures. These authors found that even after complete inactivation, the loss in heme fluorescence was approximately 50% and concluded that degradation of half the heme was sufficient for complete inactivation. Our finding on the formation of altered heme products that have absorbance properties similar to those of heme probably explains the discrepancy in the heme measurements.

The dissociable heme product, which could be separated from the nNOS protein under acidic conditions, contained an intact porphyrin ring as it exhibited a heme chromophore with a Soret maximum of 405 nm. ESI-LC-MS analysis of the

dissociable heme product produced a molecular ion at m/z 775.3, which is consistent with the mass of an adduct of heme plus NAA minus the hydrazine group. Moreover, collision-activated decomposition of the molecular ion showed heme as a fragment ion and confirmed that intact heme is a part of this adduct. These results are consistent with a mechanism of formation involving the oxidation of the hydrazine of NAA to generate a guanidino carbon radical, which subsequently attacks the heme (Fig. 8), analogous to that proposed for the reaction of hemoproteins with aryl- and alkyl-hydrazines (Augusto et al., 1982; Ator et al., 1987). The inactivation of nNOS by aminoguanidine or diaminoguanidine, both of which are hydrazine-containing guanidino compounds, has also been demonstrated to cause the formation of dissociable heme adducts that possess the Soret maximum of 405 nm (Jianmongkol et al., 2000), similar to that found for NAA. These results suggest that the formation of dissociable heme products during the inactivation of nNOS by NAA, aminoguanidine, or diaminoguanidine is likely to proceed via a common mechanism. To assess this possibility, further characterization of the molecular structure of these adducts is required.

The formation of dissociable heme products has been demonstrated for the reaction of NOS with N^{ω} -allyl-L-arginine, N^5 -(1-iminoethyl)-L-ornithine, and aminoguanidine (Zhang et al., 1997; Fast et al., 1999; Jianmongkol et al., 2000). The inactivation of inducible NOS by N^5 -(1-iminoethyl)-L-ornithine has been shown to result in the oxidation of the heme prosthetic group to biliverdin (Fast et al., 1999), and the inactivation of nNOS by N^{ω} -allyl-L-arginine generates allylated heme adducts (Zhang et al., 1997). In contrast, aminoguanidine-mediated inactivation of nNOS has been found to cause covalent alteration of the heme to products that are dissociable from the protein, but the structures have not been elucidated (Jianmongkol et al., 2000).

The current study also demonstrated that the inactivation of nNOS by NAA results in the formation of altered heme products that are irreversibly bound to the protein. Limited trypsinolysis in conjunction with a recently developed ECL assay (Vuletich and Osawa, 1998) was used to establish that the heme is irreversibly bound to a site in the oxygenase domain of the protein. The ECL assay detects the irreversibly bound heme adduct because of its inherent peroxidase activity and indicates that the heme irreversibly bound to nNOS protein is intact and redox active. Because the oxygenase domain contains the active site of the enzyme, including an appropriate binding site for heme, these results further indicate that a covalent bond between the heme and an active site amino acid may be involved. Attempts to more fully characterize the site of heme attachment were unsuccessful

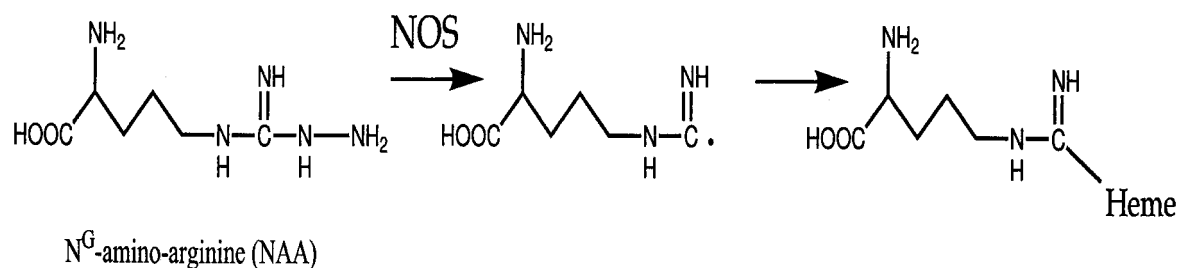


Fig. 8. Proposed mechanism for formation of the major dissociable heme adduct after treatment of nNOS with N^G -amino-L-arginine.

because of the unstable nature of the heme-peptides. Irreversibly bound heme adducts formed during the inactivation of nNOS with aminoguanidine have also been mapped to the oxygenase domain of the protein (Jianmongkol et al., 2000). To our knowledge, this report (Jianmongkol et al., 2000) and the current study are the only two cases where irreversibly bound heme adducts of nNOS have been demonstrated to form. However, examples of this reaction have been established for other hemoproteins, such as myoglobin (Catalano et al., 1989; Osawa et al., 1991), hemoglobin (Kindt et al., 1992), and liver microsomal P450 cytochromes (Osawa and Pohl, 1989; Yao et al., 1993). These alterations of nNOS caused by NAA may be biologically relevant, because of the recent finding that suicide-inactivated nNOS in HEK 293 cells is selectively degraded by the proteasome (Noguchi et al., 2000). Likewise, suicide-inactivated liver microsomal P450 cytochromes have also been shown to be degraded by the proteasome (Correia et al., 1992a; Korsmeyer et al., 1999; Wang et al., 1999), and this process seems to target selectively liver microsomal P450 cytochromes containing an irreversibly bound heme (Correia et al., 1992b; Tierney et al., 1992). In contrast, the formation of dissociable heme adducts (Tierney et al., 1992) or "heme stripping" to form apoprotein (Bornheim et al., 1987; Correia et al., 1992a) or covalent alteration of the protein (Lunetta et al., 1989) all fail to enhance proteolysis of liver microsomal P450 cytochromes. Collectively, these results suggest that irreversibly bound heme adducts, formed during metabolism-based inactivation of nNOS, may be specifically targeted for degradation by the proteasome in vivo, similar to what has been observed for the liver microsomal P450 cytochromes. This study is the first to provide evidence that irreversibly bound heme adducts of nNOS can occur in vivo.

The nNOS in GH₃ pituitary cells has been described previously as refractory to metabolism-based inactivation (Cooper et al., 1998). Conversely, the current study has established that nNOS in transfected HEK 293 cells is susceptible to metabolism-based inactivation by NAA. We demonstrated that the NAA-mediated loss of nNOS activity in these cells was time- and concentration-dependent, and required metabolically active enzyme. In addition, the inactivation of nNOS by NAA could be attenuated in the presence of the natural substrate, L-arginine, suggesting an active site-directed event. We also discovered that the NAA-mediated inactivation of nNOS in these cells was primarily due to the covalent alteration of the heme prosthetic group to products that are highly similar to those observed in vitro. Moreover, this heme alteration accounted for as much as 82% of the NAA-mediated activity loss. Thus, it seems from these data that the inactivation of nNOS by NAA in vivo is likely to occur via a mechanism similar to that observed in vitro. Based on these findings, we expect that NAA can be used as a tool to study the mechanism of the proteasomal degradation of nNOS in vivo.

The inactivation and enhanced proteolytic degradation of nNOS in vivo would result in a sustained deficit in the production of NO. This deficit may be corrected by the synthesis and assembly of new active dimeric nNOS, by assembly of a pool of inactive monomeric nNOS, or perhaps by other as yet unidentified mechanisms. Thus, NAA can be used as a molecular tool to characterize further the complex actions of NOS inhibitors on biological systems.

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Address correspondence to: Yoichi Osawa, Department of Pharmacology, The University of Michigan Medical School, 1301 Medical Science Research Building III, Ann Arbor, MI 48109-0632. Email: osawa@umich.edu
