Peroxynitrite Induces Destruction of the Tetrahydrobiopterin and Heme in Endothelial Nitric Oxide Synthase: Transition from Reversible to Irreversible Enzyme Inhibition[†]

Weiguo Chen,[‡] Lawrence J. Druhan,[‡] Chun-An Chen,[‡] Craig Hemann,[‡] Yeong-Renn Chen,[‡] Vladimir Berka,[§] Ah-Lim Tsai,[§] and Jay L. Zweier*,[‡]

[‡]*Davis Heart and Lung Research Institute, Division of Cardiovascular Medicine, Department of Internal Medicine, College of Medicine, The Ohio State University, Columbus, Ohio 43210, and* [§]*Division of Hematology, Department of Internal Medicine, University of Texas Health Science Center at Houston, Houston, Texas 77030*

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ABSTRACT: Endothelial nitric oxide synthase (eNOS) is an important regulator of vascular and cardiac function. Peroxynitrite ($ONOO^-$) inactivates eNOS, but questions remain regarding the mechanisms of this process. It has been reported that inactivation is due to oxidation of the eNOS zinc-thiolate cluster, rather than the cofactor tetrahydrobiopterin (BH_4); however, this remains highly controversial. Therefore, we investigated the mechanisms of $ONOO^-$-induced eNOS dysfunction and their dose dependence. Exposure of human eNOS to $ONOO^-$ resulted in a dose-dependent loss of activity with a marked destabilization of the eNOS dimer. HPLC analysis indicated that both free and eNOS-bound BH_4 were oxidized during exposure to $ONOO^-$; however, full oxidation of protein-bound biopterin required higher $ONOO^-$ levels. Additionally, $ONOO^-$ triggered changes in the UV/visible spectrum and heme content of the enzyme. Preincubation of eNOS with BH_4 decreased dimer destabilization and heme alteration. Addition of BH_4 to the $ONOO^-$-destabilized eNOS dimer only partially rescued enzyme function. In contrast to $ONOO^-$ treatment, incubation with the zinc chelator TPEN with removal of enzyme-bound zinc did not change the eNOS activity or stability of the SDS-resistant eNOS dimer, demonstrating that the dimer stabilization induced by BH_4 does not require zinc occupancy of the zinc-thiolate cluster. While $ONOO^-$ treatment was observed to induce loss of Zn binding, this cannot account for the loss of enzyme activity. Therefore, $ONOO^-$-induced eNOS inactivation is primarily due to oxidation of BH_4 and irreversible destruction of the heme/heme center.

Endothelial nitric oxide synthase (eNOS)[1] is a homodimeric enzyme found in many tissues and catalyzes the NADPH-dependent conversion from L-arginine to L-citrulline and nitric oxide (NO). NO is involved in many physiological processes, including regulation of vascular tension, angiogenesis, and inflammation (*1*). Abnormalities in NO production by the vascular endothelium result in endothelial dysfunction and underlie many disease pathologies that are associated with oxidant stress such as hypertension, diabetes, aging, postischemic injury, and atherosclerosis (*2*). As such, it is not surprising that multiple mechanisms and multiple steps regulate eNOS activity, including Ca^{2+}/calmodulin binding, substrate and cofactor availability, posttranslational modification, protein−protein interaction, gene expression, and protein localization (*3−6*).

Endothelial NOS, like all mammalian NOS isoforms, is composed of two domains. The C-terminal reductase domain contains FAD and FMN, along with the NADPH binding site. The N-terminal oxygenase domain contains the arginine binding site, heme group, and tetrahydrobiopterin (BH_4). The reaction mechanism is composed of two sequential steps that involve electron transfer from NADPH through the flavins to the heme iron, followed by oxygen binding and activation and subsequent substrate monooxygenation. BH_4 is critical for the formation of the activated oxygen species necessary for substrate monooxygenation, and biochemical studies have demonstrated that electron transfer from the reductase domain to the oxygenase domain is an intermonomer event, and as such NOS activity requires the association of two NOS monomers (*7*). While it is clear that the structure of the enzyme influences its activity, questions remain regarding the specific molecular mechanisms of the formation of an active NOS dimer (*8, 9*).

In addition to its involvement in the catalytic activity of the enzyme, the fully reduced biopterin is known to stabilize the eNOS dimer. Low-temperature SDS−PAGE (LT-PAGE) studies have shown that eNOS purified from a yeast expression system was mainly monomer, and that incubation with BH_4 and L-arginine lead to a pronounced shift toward eNOS dimers (*10*). Although the enzyme can be purified as the BH_4-free dimer from various expression systems, there is strong biochemical evidence indicating that the addition of BH_4, to either monomeric eNOS or preformed dimer, greatly stabilizes the dimeric structure of the enzyme (*11−14*). The heme group of eNOS has also been reported as a necessary component for eNOS dimer formation. Deletion of the amino acid residues involved in the heme ligation inhibited the formation of dimeric eNOS (*14*). In support of this hypothesis, heme release was detected during eNOS dimer dissociation by temperature-induced denaturation (*13*). However, the relationship between eNOS dimer formation and heme assembly remains uncertain.

*To whom correspondence should be addressed. Phone: 614-247-7788. Fax: 614-292-8778. E-mail: jay.zweier@osumc.edu.
[1]Abbreviations: eNOS, endothelial nitric oxide synthase; TPEN, tetrakis(2-pyridylmethyl)ethylenediamine; BH_4, (6*R*)-5,6,7,8-tetrahydrobiopterin; $ONOO^-$, peroxynitrite; DTPA, diethylenetriaminepentaacetic acid; PAR, 4-(2-pyridylazo)resorcinol.

X-ray crystallography has shown that the NOS isoforms contain a zinc-thiolate cluster located at the dimer interface of the oxygenase domain (*15*, *16*). It has been reported that alteration of the zinc thiolate, either by oxidation or nitrosylation of the cysteine sulfhydryl groups or by the addition of TPEN (a zinc chelator), results in dimer dissociation and loss of activity (*17−19*). This loss of activity could be prevented by the addition of the thioredoxin/thioredoxin reductase system or by the addition of exogenous zinc (*17*, *19*). Moreover, it has been postulated that oxidation of the zinc-thiolate cluster is the predominant mechanism involved in $ONOO^-$-induced eNOS dysfunction (*18*). However, there have been contradictory reports indicating that neither chemical removal of zinc nor mutation of the cysteine residues involved in zinc binding produces an inactive enzyme (*11*, *20−22*).

$ONOO^-$ is a highly reactive oxidant formed by the reaction of superoxide and NO (*23*). An increased formation of $ONOO^-$ in vivo has been detected in disease conditions such as ischemia-reperfusion injury, sepsis, inflammation, and atherosclerosis (*24−27*), and it is clear that eNOS is involved in the generation of $ONOO^-$ under pathological conditions (*24*, *28*). NOS enzymes are known to be susceptible to inactivation by oxidants, including $ONOO^-$ (*18*, *29*), but the specific mechanism of inactivation is controversial and uncertain. Therefore, we investigated the mechanisms of eNOS dimer destabilization and inactivation induced by $ONOO^-$. We find that after free BH_4 is consumed by $ONOO^-$ oxidation, further oxidation of protein-bound BH_4 initiates eNOS dimer destabilization and a $ONOO^-$-induced disruption of the eNOS heme. Furthermore, we observe that the dimer stabilization induced by BH_4 is not dependent upon the presence of zinc in the zinc-thiolate cluster. While addition of fresh BH_4 can reconstitute the $ONOO^-$-destabilized eNOS dimer, the $ONOO^-$-induced heme disruption is not reversed, and as such the SDS-resistant dimer is not fully active.

MATERIALS AND METHODS

Materials. Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) was obtained from Molecular Probes Inc. (Eugene, OR). Hemin was purchased from Frontier Scientific Co. (Lagon, UT). CaM, NADPH, L-arginine, BH_4, *N*-nitro-L-arginine methyl ester (L-NAME), and $ONOO^-$ were purchased from Upstate Cell Signaling Solutions (Lake Placid, NY); the $ONOO^-$ concentration was determined by absorbance at 302 nm ($E_{302} = 1.67$ mM^{-1} cm^{-1}). The $ONOO^-$ stock, in 0.3 M NaOH, was diluted with 10 mM NaOH to the appropriate concentrations just before addition to the protein samples. Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

Protein Purification. Human eNOS cDNAs encoding the full protein sequence and the oxygenase domain (amino acids 1−491) were each overexpressed in yeast, and the recombinant protein was purified either in the presence of BH_4 or in the absence of BH_4 as described previously (*30*) with modifications (*31*). After purification, any excess BH_4 was removed by ultrafiltration.

Detection of SDS-Resistant eNOS Dimers. The full-length eNOS SDS-resistant monomer−dimer equilibrium was assayed using low-temperature SDS−PAGE (LT-PAGE) under reducing conditions as described previously with modifications (*32*). After treatment, eNOS samples were subjected to low-temperature SDS−PAGE in reducing conditions (with 2.5%

2-mercaptoethanol) to resolve the dimer and monomer. The protein bands were visualized by Coomassie blue staining, and heme content was detected as peroxidase activity of each protein band (see below). The signal intensity of each band was quantified using an Alphalmager high-performance gel documentation and image analysis system, Model 3300 (Alpha Innotech Co., San Leandro, CA).

Heme Staining Assay. After treatment, samples were subjected to LT-PAGE. The gels obtained from LT-PAGE were subjected to heme staining with 3,3′-dimethoxybenzidine/H_2O_2 according to the published method with modifications (*13*). Gels were washed with methanol/sodium acetate (0.25 M, pH 5.0; 3:7 v/v) for 10 min, and subsequently incubated in the dark for 20 min in a freshly prepared solution, containing 7 parts of 0.25 M sodium acetate, pH 5.0, and 3 parts of 6 mM 3,3′-dimethoxybenzidine dihydrochloride in methanol. Gels were developed for 60 min by adding 60 mM H_2O_2 and then washed with water/methanol/acetic acid (8:1:1 v/v) for 30 min prior to densitometric analysis of heme staining.

Measurement of eNOS Activity. The release of NO generated by eNOS was measured by the oxyhemoglobin to methemoglobin conversion assay following the procedures developed by Sheta et al. and Stuehr et al. with minor modification (*33*, *34*). The reaction system contained 5 μM oxyhemoglobin, 4 μg of purified human eNOS, 0.2 mM DTT, 1 mM $CaCl_2$, 1 μg/mL CaM, 5 μM BH_4, and 200 μM NADPH in 50 mM HEPES buffer, pH 7.4, in a total volume of 100 μL. The reaction was initiated by the addition of 100 μM L-arginine. NO generation was measured by monitoring the change in absorbance $A_{401} − A_{411}$ and a millimolar absorbance coefficient of 38 mM^{-1} cm^{-1}. For activity measurements of the $ONOO^-$-treated eNOS, the appropriate volume of diluted $ONOO^-$ was added, and the samples were incubated on ice for 10 min. Since the half-life of $ONOO^-$ at neutral pH values is about 1 s, it is completely decomposed prior to addition to the assay mixture (*35*). For activity measurements with TPEN, a 1.25 mM TPEN solution was prepared in water, and this solution was used for the preparation of the reaction buffer above such that the final TPEN concentration was 1 mM.

Analysis of Free and eNOS-Bound BH_4. Total BH_4 content in eNOS was measured using HPLC analysis. After eNOS or free BH_4 was treated with $ONOO^-$, eNOS-bound BH_4 and free BH_4 were extracted with buffer containing 6.5 mM dibasic sodium phosphate, 6 mM citric acid, 5 mM dithioerythritol, 2.5 mM DTPA, 3 mM 1-octanesulfonic acid, and 10% methanol. This buffer is able to extract and maintain the redox state of BH_4. The HPLC system consisted of a CoulArray system from ESA Analytical, Ltd. (Chelmsford, MA), with automated gradient controller, refrigerated auto sampler, and ESA software for data collection and analysis. The chromatographic separation was carried out using a Toso Haas 5 μm ODS-80TM reverse-phase column (4.6 mm × 25 cm) with flow rate of 1.3 mL/min. Tetrahydrobiopterin was electrochemically detected with the analytical electrodes set at 0.05 and 0.4 V. The guard cell was set at 0.8 V.

Heme and Zinc Quantitation. Heme content of eNOS was determined by the formation of pyridine hemochromogen (*36*). Briefly, 80 μg of purified eNOS was added to a solution of 0.15 M NaOH and 1.8 M pyridine, and the spectrum was recorded. The sample was then reduced with dithionite, and a second spectrum was recorded. The total heme content was calculated from the difference spectrum ($A_{556−538nm}$), using a millimolar absorbance coefficient of 24 mM^{-1} cm^{-1}. Free zinc was measured using the
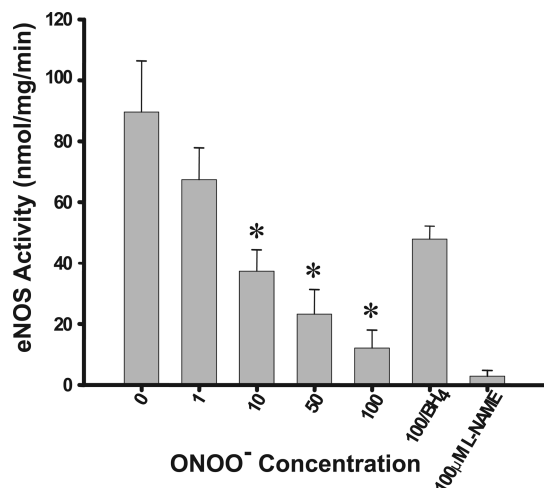
FIGURE 1: Inactivation of eNOS activity by ONOO⁻ and the protective effect of BH₄. Purified human eNOS (0.1 μg/μL) in 50 mM HEPES, pH 7.4, was treated with the indicated concentrations of ONOO⁻. After incubation (45 min on ice) eNOS activity was measured as described in the Materials and Methods. The values of eNOS activity presented are obtained from the average of six independent assays (*, $P < 0.05$).

4-(2-pyridylazo)resorcinol (PAR) assay as described previously (*18*). Zinc, iron, and copper content were also measured by ICP-MS. For ICP-MS 50 μg of eNOS was added to 50 mM KH₂PO₄, 150 mM NaCl, 0.6 mM dithiothreitol, and 2 mM DTPA (to chelate adventitious metals) at pH 7.4, in a total volume of 150 μL. Three sets of samples were analyzed: an untreated sample, a sample treated with 100 μM ONOO⁻, and a sample treated with 1 mM TPEN. Each sample was incubated on ice for 2 h, then filtered through 3000 MW cutoff Microcon spin columns, and washed twice with the treatment buffer. The filtrates and washed protein samples were then assayed for Zn, Fe, and Cu by ICP-MS as described previously (*37*).

UV/Vis Spectroscopy. Optical absorbance was measured in a Cary 300 double beam spectrophotometer from Varian, Inc. (Palo Alto, CA), using 1 cm path length cuvettes at room temperature (23 °C).

Statistical Analysis. Data are expressed as mean $\pm$ SE experiments with given number or at least three independent experiments. Differences in mean value were analyzed by Student's *t* test, and a value of $P < 0.05$ was considered as statistically significant.

RESULTS

ONOO⁻ Inactivates eNOS, and This Is Inhibited by Exogenous BH₄. To investigate the effect of ONOO⁻ on the activity of eNOS, we treated recombinant human eNOS with increasing concentrations of ONOO⁻ and subsequently measured the rate of NO production. The activity of untreated eNOS purified from yeast expression system was 92 $\pm$ 18.4 nmol mg^{-1} min^{-1} (Figure 1), which is consistent with previously reported activities for the endothelial isoform (*10, 12*). Incubating eNOS with ONOO⁻ resulted in a dose-dependent loss of eNOS activity, in agreement with previously published results (*18*). The NO generation rate was decreased to 16.5 $\pm$ 6.5 nmol mg^{-1} min^{-1} when eNOS was treated with 100 μM ONOO⁻. Although preincubation of eNOS with 100 μM BH₄ did not fully protect the enzyme from oxidative damage, it did provide some protection, decreasing the ONOO⁻ inactivation by ~70%.
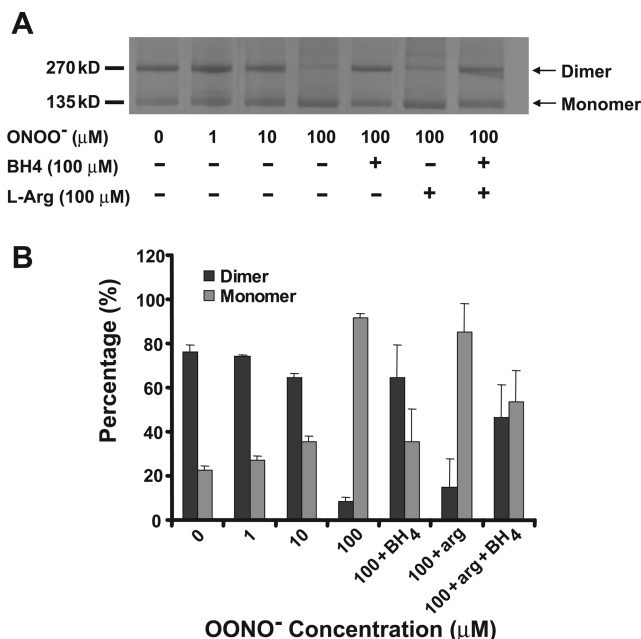

FIGURE 2: ONOO⁻-induced eNOS dimer destabilization and the protective effects of BH₄. (A) Purified human eNOS (0.1 μg/μL) in 50 mM HEPES, pH 7.4, was preincubated with L-arginine (100 μM) or BH₄ (100 μM) at 0 °C for 30 min. The enzyme was treated with the indicated concentration of ONOO⁻ for 45 min at 0 °C. The entire mixture was then subjected to LT-PAGE, and the protein bands were visualized by Coomassie blue staining. (B) The ratio of eNOS dimer/monomer in (A) was quantitated by digital densitometry as described in the Materials and Methods. The data analyses were obtained from the average of six independent experiments.

To begin to elucidate the molecular mechanism underlying the ONOO⁻-induced eNOS inactivation, we sought to determine if exposure to ONOO⁻ induced destabilization of the eNOS dimer. As shown in Figure 2A, low-temperature SDS−PAGE can be used to detect changes in dimer stability, detecting both the eNOS monomer with a molecular mass of 135 kDa and the homodimer with a molecular mass of 270 kDa. When saturated with BH₄, eNOS demonstrated approximately 80% SDS-resistant dimer. Incubation of eNOS with ONOO⁻ destabilized the eNOS dimer in a concentration-dependent manner. The percentage of SDS-resistant eNOS dimer decreased to less than 10% when eNOS was incubated with 100 μM ONOO⁻ for 45 min at room temperature, with a concomitant increase of apparent monomer. A similar pattern of ONOO⁻-induced dimer destabilization was also observed using recombinant bovine eNOS expressed in *Escherichia coli* (data not shown). Preincubation of eNOS with 100 μM BH₄ significantly protected the eNOS dimer from the destabilization induced by 100 μM ONOO⁻. The SDS-resistant dimer content of ONOO⁻-treated eNOS with or without BH₄ preincubation was 64% and <10%, respectively. However, we did not observe a protective effect from 100 μM L-arginine. Only 14% SDS-resistant dimer was observed when eNOS was pretreated with L-arginine and then treated with ONOO⁻. Furthermore, there was no synergistic protective effect of BH₄ and L-arginine (Figure 2).

BH₄ Bound to eNOS Is a Target of Oxidation by ONOO⁻. Previously reported results indicated that BH₄ inhibited eNOS dimer dissociation induced by ONOO⁻ (*19*). Pure BH₄ can react with ONOO⁻ to form an intermediate, the BH₃ radical, and subsequently BH₂ (*38*), and studies have shown that autoxidation of BH₄ in eNOS resulted in the loss of enzymatic
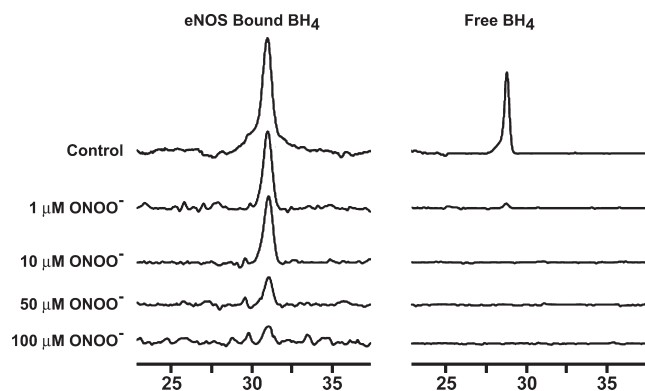
FIGURE 3: $ONOO^-$-induced oxidation of BH_4 in eNOS or free BH_4 as measured by HPLC with electrochemical detection. Purified eNOS (0.15 $\mu g/\mu L$) or free BH_4 (1 μM) was incubated in 100 μL of 50 mM HEPES, pH 7.4, with different concentrations of $ONOO^-$ as indicated for 45 min. 80 μL of BH_4 extraction buffer was added into the solution and centrifuged for 15 min at 16000g. The supernatant was filtered, and aliquots (25 μL) of the filtrate were subjected to HPLC analysis.
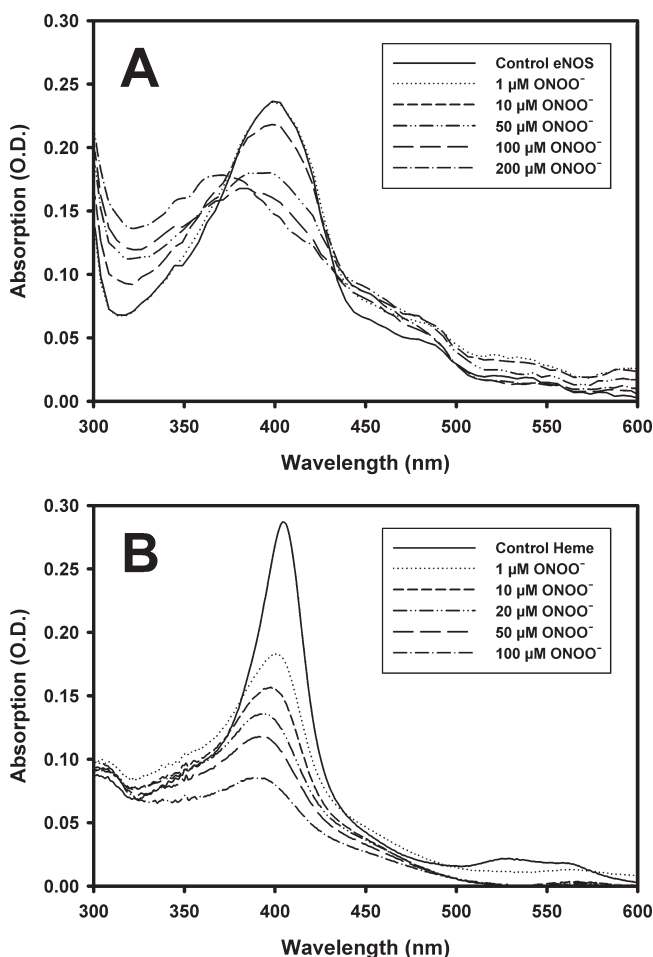

FIGURE 4: $ONOO^-$-induced changes in the UV/vis spectra of eNOS and pure heme. (A) eNOS (0.24 $\mu g/\mu L$) in 50 mM HEPES, pH 7.4, was treated with various concentrations of $ONOO^-$ and incubated for 10 min at room temperature, and the UV/vis spectra were recorded. (B) Same as (A) except that the buffer included 5% pyridine, and the eNOS was replaced with pure iron protoporphyrin (2 μM).
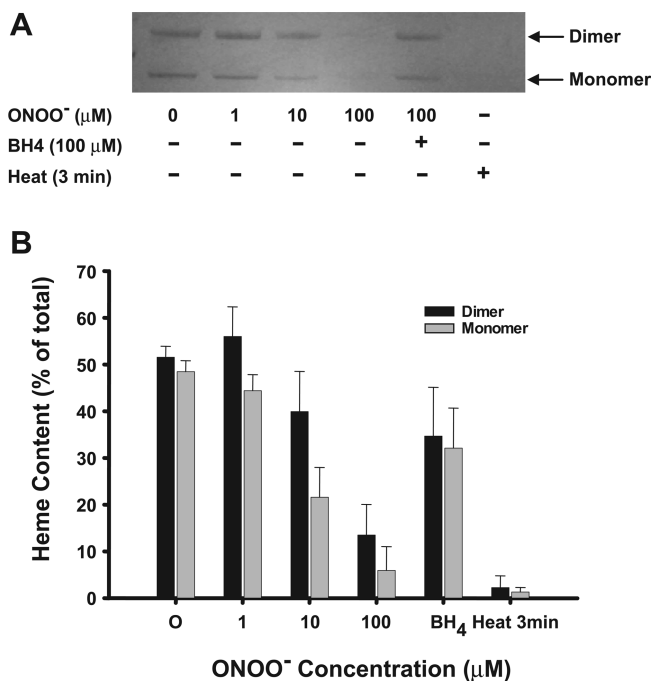
FIGURE 5: Detection of heme release from $ONOO^-$-treated eNOS by heme staining. (A) Purified human eNOS (0.1 $\mu g/\mu L$) was incubated in HEPES, pH 7.4, with the indicated concentration of $ONOO^-$ for 45 min. The samples were subjected to LT-PAGE and heme staining as described in the Materials and Methods. (B) The heme content was quantitated by digital densitometry as described in the Materials and Methods and represented as the percent of the total eNOS heme detected in either the monomer or dimer.

room temperature, and BH_4 was then extracted from the reaction mixtures followed by HPLC analysis. A chromatogram representing typical results is shown in Figure 3; BH_4 had a retention time of ~30 min. As shown in Figure 3, the amount of BH_4 extracted from $ONOO^-$-treated eNOS gradually decreased as the dosage of $ONOO^-$ was elevated. Only trace amounts of BH_4 (~8%) were still bound to eNOS at 100 μM $ONOO^-$. We also investigated the $ONOO^-$-induced oxidation of free BH_4. Pure BH_4 (1 μM) was incubated with various concentrations of $ONOO^-$, and the results were detected by HPLC. As shown in Figure 3, addition of 1 μM $ONOO^-$ oxidized more than 95% of free BH_4. Thus, free BH_4 is oxidized in essentially a 1:1 stoichiometric ratio by addition of $ONOO^-$ (*38*).

Effect of $ONOO^-$ on the Heme Center of eNOS and Hemin (Iron Protoporphyrin IX). The NOS heme center has been implicated as critical to eNOS dimer formation, and deletion of the cysteine involved in heme binding resulted in the inhibition of eNOS dimer association (*14*). To investigate how $ONOO^-$ affects the heme center of eNOS, we followed the absorption changes of the heme as eNOS was exposed to $ONOO^-$. As shown in Figure 4A, recombinant eNOS demonstrated a typical NOS high-spin heme absorption with a Soret band at 398 nm. As the $ONOO^-$ concentration was increased, the heme absorption at 398 nm decreased. Approximately 70% of eNOS-bound heme absorption was bleached by the addition of 100 μM $ONOO^-$ (Figure 4A). Similar spectral changes were observed using eNOS and eNOS oxygenase domain purified from *E. coli*. Moreover, when pure iron protoporphyrin IX was incubated with increasing concentrations of $ONOO^-$, a dose-dependent decrease in heme absorption at the Soret γ band (λ_{max} = 405 nm) was also observed (Figure 4B). More than 85% of the iron protoporphyrin IX absorption was bleached when

activity (*31*). To directly address whether or not BH_4 bound to eNOS is a target for $ONOO^-$ oxidation, 15 μg of eNOS was incubated with various concentrations of $ONOO^-$ for 45 min at
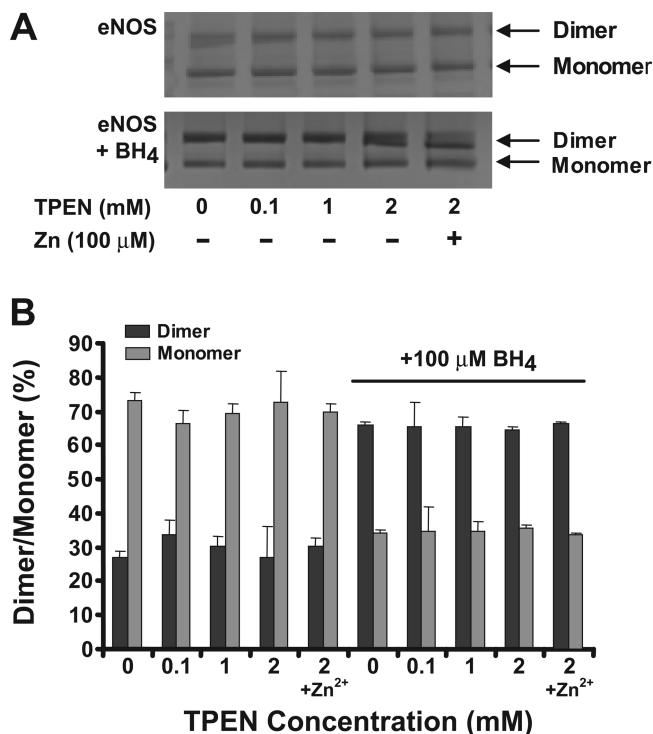
FIGURE 6: Effect of TPEN, Zn^{2+}, and BH_4 on eNOS dimerization. (A) Purified human eNOS ($0.1 \mu g/\mu L$) in 50 mM HEPES, pH 7.4, was treated with various concentrations of TPEN for 45 min in the presence or absence of 100 μM Zn^{2+}. The mixture was subjected to LT-PAGE, and the protein bands were visualized by Coomassie blue staining. (B) Same as (A) except the eNOS was preincubated with or without BH_4 (100 μM) prior to addition of TPEN and Zn^{2+}. The ratios of eNOS dimer/monomer were quantitated by digital densitometry as described in the Materials and Methods.

FIGURE 7: Effects of BH_4 postaddition on the restoration of eNOS dimer stability and the enzymatic activity of eNOS. (A) eNOS ($0.2 \mu g/\mu L$) in HEPES, pH 7.4, was treated with 100 μM $ONOO^-$. BH_4, L-arginine, or both were then added to the $ONOO^-$-treated eNOS. The mixtures were incubated for 30 min and then subjected to LT-PAGE and staining with Coomassie blue. (B) Samples were the same as in (A), except that aliquots of each mixture were withdrawn for measurement of eNOS activity as described in the Materials and Methods.

exposed to 100 μM $ONOO^-$, indicating that free iron protoporphyrin IX is more sensitive to $ONOO^-$ treatment than eNOS-bound heme (Figure 4B).

To investigate the role of the observed $ONOO^-$-induced heme bleaching in the eNOS dimer formation/dissociation, $ONOO^-$-treated enzyme was subjected to LT-PAGE, and heme content was determined. For native recombinant eNOS, heme can be detected in both SDS-resistant dimer and SDS-induced monomer (Figure 5). Incubating eNOS with various concentrations of $ONOO^-$ at room temperature decreased the detectible heme content of both monomer and dimer (as detected on low-temperature SDS−PAGE) in a dose-dependent manner. Heme was almost completely undetectable after treatment with 100 μM $ONOO^-$. Preincubation of eNOS with 100 μM BH_4 for 30 min significantly inhibited $ONOO^-$-induced heme loss. Heating eNOS (95 °C) for 3 min in SDS−PAGE buffer resulted in complete loss of detectible heme from eNOS (Figure 5A). Notably, we detected a greater $ONOO^-$-induced heme loss from the monomer compared to SDS-resistant dimer of eNOS at all $ONOO^-$ concentrations tested. It should be noted that our heme staining method detects the inherent peroxidase activity of the heme, and as such any detectable loss of activity that migrates with the full-length eNOS on SDS−PAGE can be interpreted as release of the heme from the enzyme or disruption of the bound heme.

BH_4 Does Not Require Zinc Binding for eNOS Dimer Stabilization. Previous studies have indicated that the zinc-thiolate (ZnS_4) cluster, identified by examination of the X-ray crystal structure of the oxygenase domain dimer, plays an
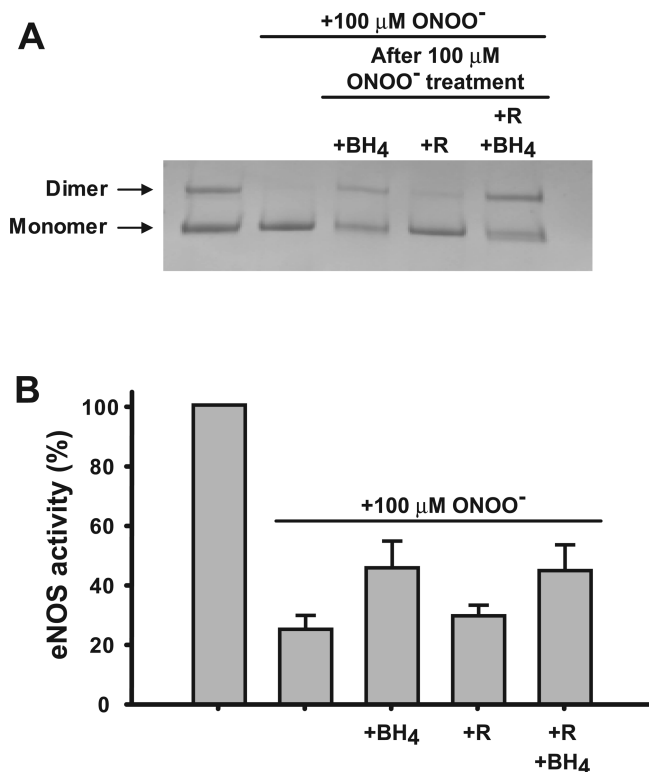
important role in eNOS structure and function (*9, 15*), and the 1:2 zinc to iron stoichiometry of eNOS zinc binding has been demonstrated (*39*). Our studies have indicated that BH_4 contributes to eNOS dimer stabilization and inhibits the dimer destabilization induced by $ONOO^-$. It is important to clarify whether the zinc-thiolate cluster is the predominant factor for eNOS dimer stabilization and whether both BH_4 and the zinc-thiolate cluster can affect eNOS dimer formation in a synergistic way.

In order to confirm $ONOO^-$-induced zinc release, we exposed our purified eNOS to 100 μM $ONOO^-$ and measured free zinc with the PAR assay (*18*). For our recombinant eNOS, this treatment resulted in the release of ~0.35 nmol of zinc for each nmol of eNOS dimer; thus it is clear that $ONOO^-$ treatment does induce zinc loss. Next we exposed eNOS to the selective zinc-chelating agent TPEN, in the absence or presence of BH_4. Incubation of eNOS with 1 mM TPEN for 2 h on ice did not alter the activity of the enzyme. We measured the amount of zinc removed by TPEN treatment using ICP-MS and found that ~80% of the eNOS-bound zinc was removed by the TPEN treatment. To determine if incubation with TPEN altered the stability of the dimeric structure of eNOS, we used low-temperature SDS−PAGE. As shown in Figure 6A (upper panel), BH_4-free eNOS was only 27% dimer, and incubation of eNOS with TPEN did not cause a detectable change in dimer stabilization. Moreover, incubation with 100 μM Zn^{2+} did not alter the SDS-resistant eNOS dimer structure. These results are not consistent with those previously reported (*18*). However, 100 μM BH_4 did

significantly enhance eNOS dimer stabilization (Figure 6A, lower panel), consistent with previously published reports (10, 12–14). Incubation of eNOS with 100 μM BH$_4$ increased the dimer percentage from ~27% to ~65%, irrespective of the presence or absence of TPEN, and like in the BH$_4$ free enzyme, exogenous Zn^{2+} had no effect on eNOS dimer stabilization in the absence or presence of 100 μM BH$_4$ (Figure 6B).

BH$_4$ Rescues ONOO$^-$-Destabilized eNOS (SDS-Resistant Dimer) but Not eNOS Function. Our current and previously published work (29) demonstrates that BH$_4$ is oxidized by ONOO$^-$ leading to inhibition of NOS-dependent NO formation. Additionally, preincubation of eNOS with excess BH$_4$ can significantly protect eNOS structure and function against ONOO$^-$ damage. It is of interest to determine whether BH$_4$ functions just as an ONOO$^-$ scavenger or if it can restore the stability of the eNOS dimer and rescue ONOO$^-$-induced decreases in enzyme activity. To address this issue, the eNOS dimer stability and enzymatic activity were measured before and after subsequent incubation with BH$_4$, L-arginine, or both. As shown in Figure 7A, incubation of 100 μM BH$_4$ with ONOO$^-$-treated eNOS resulted in recovery of the SDS-resistant eNOS dimer structure, and this recovery was mildly synergistic with arginine; however, incubation with L-arginine (100 μM) alone did not recover the eNOS dimer. Although BH$_4$ addition did rescue some ONOO$^-$-induced loss of eNOS activity, unlike the dimer stabilization this recovery of activity was not complete. The activity of ONOO$^-$-treated eNOS was increased from 25%, to 46% (+BH$_4$), to 29% (+Arg), and to 45% (+BH$_4$; +Arg) (Figure 7B), as such BH$_4$ is only able to restore about one-third of the ONOO$^-$-induced loss of eNOS activity.

DISCUSSION

In the current studies, we have demonstrated that ONOO$^-$ causes eNOS dimer destabilization via the oxidation of BH$_4$. Exogenous BH$_4$ can inhibit the ONOO$^-$-mediated eNOS dimer destabilization, mainly by acting as a scavenger of the oxidant. Furthermore, our studies indicate that BH$_4$ is the predominant factor for SDS-resistant eNOS dimer stabilization when compared with the effects induced by L-arginine binding and/or by influences of the presence of zinc in the zinc-thiolate cluster. We have also demonstrated the novel finding that ONOO$^-$ treatment causes a major alteration in heme structure rendering the NOS heme negative for peroxidase-activity-based staining. Finally, although BH$_4$ can reconstitute the ONOO$^-$-destabilized SDS-resistant eNOS dimer, the re-formed dimer is not fully functional.

ONOO$^-$ is a highly reactive oxidant formed in biological systems when superoxide and NO are produced simultaneously, and the physiological flux of ONOO$^-$ has been estimated to be approximately 0.2–0.4 μM/s (23, 40). The majority of NO in biological systems is generated from the various NOS isoforms, and it is clear that the eNOS produces superoxide under certain conditions (41, 42). Indeed, under conditions of limited L-arginine or BH$_4$ depletion the NOS isozymes generate both NO and superoxide simultaneously (41, 43–45). Additionally, superoxide is produced from a number of different sources under various physiologic and pathophysiologic conditions (46). The ONOO$^-$ formed in vivo has a wide array of tissue damaging effects ranging from lipid peroxidation, inactivation of enzymes and ion channels via protein oxidation and nitration, and inhibition of mitochondrial respiration (23). It has been reported that ONOO$^-$ inactivates NO generation from all of the NOS isoforms (18, 29,

47, 48); however, the mechanisms by which this inactivation occurs have been controversial.

Peroxynitrite Destabilizes the eNOS Dimer via Oxidation of BH$_4$, Not Removal of Zinc from the Zinc-Thiolate Cluster. It has been reported that ONOO$^-$ caused eNOS dimer dissociation by breaking the putative zinc-thiolate cluster that is located at the dimer interface and that this dimer dissociation effectively uncouples NADPH oxidation from NO production leading to loss of NOS activity (18). Additionally, these authors postulate that the oxidation of the zinc-thiolate cluster is the predominant factor in the observed ONOO$^-$-induced uncoupling of eNOS rather than oxidation of BH$_4$. However, there is also structural and biochemical evidence demonstrating that BH$_4$ is involved in the stabilization of the NOS dimer. X-ray crystal structures of the oxygenase domains from many NOS isoforms demonstrate that the BH$_4$ binding site is located at the dimer interface, situated in a pocket composed of several conserved residues (9, 15). Although BH$_4$ is not strictly required for dimerization, there is a marked stabilization of the active dimer with BH$_4$ binding (11–14, 49, 50). From our HPLC analysis, and from previously published data (38, 51–53), it is clear that BH$_4$, both when it is free in solution and when it is protein bound, is a target for oxidation. It is well established that the fully reduced biopterin is required for the coupling of NADPH oxidation to the production of NO, and for suppression of superoxide generation from eNOS (41, 42). Therefore, it is clear that oxidation of BH$_4$ is instrumental in the uncoupling of NOS irrespective of the stability of the NOS dimer, and our results suggest that oxidation of BH$_4$ in eNOS is a predominant driving force in ONOO$^-$-induced SDS-resistant dimer dissociation.

Although we could detect ONOO$^-$-induced release of zinc from eNOS, in contrast to previously published work we could not detect any change in the SDS-resistant eNOS monomer: dimer ratio with either the addition of exogenous zinc or the addition of the zinc chelator TPEN. While we cannot completely rule out the involvement of the zinc-thiolate cluster in the ONOO$^-$-induced destabilization of the eNOS dimer, our data and previously published work on other NOS isoforms indicate that the direct contribution of the presence of zinc in the cluster to dimer stabilization is small relative to that provided by bound BH$_4$ and the heme. Indeed, studies have been performed on nNOS, which indicated that removal of zinc from nNOS by reversible chemical modification of the cysteine residues destabilized the SDS-resistant dimer but that this effect was blocked by the addition of BH$_4$ (11). Additionally, upon reversal of the cysteine modification, the zinc-free nNOS retained almost 100% activity.

Mutational analyses in nNOS (C331A) and eNOS (C99A) have demonstrated that although the presence of zinc in the Zn–S$_4$ cluster is not strictly required for catalysis, the structural integrity of the cluster which is altered by the mutations is important for binding BH$_4$ and/or arginine (21, 22). The C331A nNOS mutation produced an inactive enzyme, but overnight incubation with excess arginine restored activity (21), and likewise, the inactive C99A eNOS mutant was restored by overnight incubation with BH$_4$ (22). This is not surprising given the structural contiguity identified between the Zn–S$_4$ cluster and the cofactor/substrate binding sites (15). Additionally, mutation of the specific cysteine residues involved in the zinc binding of iNOS to alanine resulted in the loss of zinc but did not inhibit dimer formation or enzyme activity under normal assay conditions (20). These authors did demonstrate an inhibition of

enzyme activity in the cysteine to alanine mutants when the enzyme was preincubated at 37 °C and demonstrated an inactivation of the wild-type enzyme via an NO-driven nitrosylation of the zinc-thiolate cysteines. Additionally, the zinc content of both nNOS and eNOS has been shown to decrease in the absence of BH$_4$ (*39*). Therefore, although BH$_4$ provides a much more significant stabilization of the eNOS dimer relative to the zinc occupancy of the Zn$-$S$_4$ cluster, it is clear that the structure of this cluster is involved in the formation of the fully active conformation of the enzyme. As such, modification of this cluster, either by oxidation or nitrosylation, could provide a means by which enzyme activity is regulated by changing BH$_4$ and/or arginine binding affinity.

Peroxynitrite-Induced Heme Destruction. We have observed the novel finding that the BH$_4$ mediated ONOO$^-$-induced dimer destabilization and enzyme inactivation was accompanied by an alteration in the bound heme cofactor or its environment, as indicated by marked changes in the optical absorbance spectrum. Similar ONOO$^-$-induced changes in visible absorption spectra have been observed for both iNOS and nNOS (*47, 48*). We have demonstrated that these spectral changes are accompanied by heme loss or destruction from both the SDS-resistant eNOS dimer and monomer. Furthermore, addition of ONOO$^-$ to free hemin resulted in significant quenching. Therefore, we hypothesize that in addition to oxidizing BH$_4$ ONOO$^-$ treatment also disrupts the heme center via either an indirect mechanism involving oxidation of amino acids required for heme binding, producing heme release and subsequent destruction of the free heme, or a direct mechanism by modification of the NOS-bound heme.

The cysteine residue proximal to the heme is a good candidate target for the destruction mechanism. Indeed, the ONOO$^-$-induced heme spectral changes we observed are similar to those observed with formation of an inactive NOS P420 species generated by NO-induced dissociation of the proximal cysteine (*54*). However, our observed spectral changes are of the ferric enzyme and are not reversible by the addition of reducing agents or BH$_4$. Additionally, conversion to the P420 form has not been demonstrated to produce heme loss (*54, 55*), and the P420 form would still be expected to retain peroxidase activity (*56*). Thus, we conclude that ONOO$^-$-induced dissociation of the proximal cysteine is not the only mechanism involved in the observed heme destruction.

The observed ONOO$^-$-induced change in the heme optical absorption (bleaching) occurs irrespective of the expression system used for the recombinant enzyme and is not inhibited by the presence of bound BH$_4$ (although exogenously added BH$_4$ will act as a ONOO$^-$ scavenger and inhibit the bleaching). The extent of the observed spectral change is dependent upon the ONOO$^-$/eNOS molar ratio, with higher ratios giving more bleaching. Our data are consistent with a model similar to that proposed for the reaction of peracids with cytochromes P450 (*57, 58*), wherein destruction of the porphyrin ring (which leads to the observed bleaching of the Soret transition) is due to the reaction of excess peracid with a higher oxidation state heme. Peracids can react with P450 hemes to form the oxenoid intermediate (Fe^{IV}=O:cation radical) and subsequent reaction with another equivalent of peracid leading to heme bleaching. It has been demonstrated that heme-thiolate groups, including the iNOS heme, can react with ONOO$^-$, potentially via a ferryl-heme intermediate (*59, 60*). Thus, we hypothesize that ONOO$^-$ first reacts with the eNOS heme forming the ferryl intermediate (or other higher oxidation state heme) which then reacts with a

second equivalent of ONOO$^-$, leading to the observed heme bleaching and the loss of eNOS peroxidase activity. As expected for any P450-type enzyme, heme destruction results in eNOS inactivation (Figure 7A).

In conclusion, ONOO$^-$ oxidizes the eNOS-bound BH$_4$, triggering eNOS dimer dissociation and loss of activity. With increasing concentrations ONOO$^-$ also induces the destruction of the heme group and further irreversible loss of activity. Oxidation of the zinc-thiolate cluster with loss of zinc binding does occur, but the zinc loss does not appear to cause enzyme inactivation. Preincubation of eNOS with BH$_4$ can protect the structural integrity and enzymatic activity of eNOS from ONOO$^-$ damage via an antioxidant mechanism. Postaddition of BH$_4$ can restabilize the ONOO$^-$-destabilized eNOS dimer, but it cannot completely restore the enzymatic activity due to damage to the eNOS heme. Therefore, we propose a model of ONOO$^-$-induced inactivation of eNOS wherein at low oxidant concentrations BH$_4$ is the primary target for oxidation, leading to reversible enzyme inactivation, while at higher ONOO$^-$ concentrations, the heme or the heme site becomes susceptible to damage, leading to heme destruction and irreversible enzyme inactivation.

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