Uncoupling of eNOS by ONOO$^-$

Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid and thiols:

Implications for uncoupling endothelial nitric oxide synthase

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Tetrahydrobiopterin (BH$_4$) serves as a critical co-factor for the endothelial nitric oxide synthase (eNOS). A deficiency of BH$_4$ results in eNOS uncoupling, which is associated with increased superoxide and decreased NO$^•$ production. BH$_4$ has been suggested to be a target for oxidation by peroxynitrite (ONOO$^-$) and ascorbate has been shown to preserve BH$_4$ levels and enhance endothelial NO$^•$ production, however the mechanisms underlying these processes remain poorly defined. To gain further insight into these interactions, the reaction of ONOO$^-$ with BH$_4$ was studied using electron spin resonance (ESR) and the spin probe 1-hydroxy-3-carboxy-2, 2,5-tetramethyl-pyrrolidine (CPH). ONOO$^-$ reacted with BH$_4$ 6-10 times faster than with ascorbate or thiols. The immediate product of the reaction between ONOO$^-$ and BH$_4$ was the trihydrobiopterin radical (BH$_3^•$), which was reduced back to BH$_4$ by ascorbate while thiols were not efficient in recycling of BH$_4$.

Uncoupling of eNOS caused by peroxynitrite was investigated in cultured bovine aortic endothelial cells (BAECs) by measuring superoxide and NO$^•$ using spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH) and the NO$^•$-spin trap iron-diethylidithiocarbamate (Fe(DETC)$_2$). Bolus ONOO$^-$, the ONOO$^-$ donor SIN-1 and an inhibitor of BH$_4$ synthesis DAHP uncoupled eNOS increasing superoxide and decreasing NO$^•$ production. Exogenous BH$_4$ supplementation restored endothelial NO$^•$ production. Treatment of BAECs with both BH$_4$ and ascorbate prior to ONOO$^-$ prevented uncoupling of eNOS by ONOO$^-$.

This study demonstrates that endothelial BH$_4$ is a crucial target for oxidation by ONOO$^-$, and that BH$_4$ reaction rate constant exceeds those of thiols or ascorbate. We confirmed that ONOO$^-$ uncouples eNOS by oxidation of tetrahydrobiopterin and that ascorbate does not fully protect BH$_4$ from oxidation but recycles BH$_3^•$ radical back to BH$_4$. 
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**INTRODUCTION**

The endothelial nitric oxide synthase (eNOS) is a dimeric enzyme composed of two catalytic domains: a C-terminal reductase domain which binds NADPH, FMN and FAD and an N-terminal oxygenase domain which binds a prosthetic heme group, 5,6,7,8-terahydrobiopterin (BH$_4$), oxygen and L-arginine (1-5). The catalytic production of nitric oxide involves flavin-mediated electron transfer from C-terminal bound NADPH to the N-terminal heme center. At the heme site oxygen is reduced and incorporated into the guanidino group of L-arginine producing NO$^\cdot$ and L-citrulline (1,2,6). eNOS is only catalytically active in the dimeric form, and the ability to bind BH$_4$ is dependent on dimer formation. There is evidence that BH$_4$ promotes dimer formation, although this is controversial (7).

BH$_4$ plays a critical role in allowing electron transfer from the prosthetic heme to L-arginine. In the absence of BH$_4$ electron flow from the reductase domain to the oxygenase domain is diverted to molecular oxygen rather than to L-arginine, leading to a condition known as eNOS uncoupling (8,9) which causes production of superoxide rather than nitric oxide.

Superoxide reacts rapidly with NO$^\cdot$ to form the peroxynitrite anion (ONOO$^-$), which is a strong biological oxidant (10) known to oxidize lipids, protein, sulphydryls and DNA and to cause nitration of tyrosines (11-13). Recently it has been suggested that BH$_4$ is an important target for oxidation by ONOO$^-$ (Scheme 2) (14). Treatment of purified eNOS with ONOO$^-$ significantly decreases the ability of the enzyme to produce NO$^\cdot$ (15). Laursen et al. demonstrated that ONOO$^-$ is more potent than either superoxide or H$_2$O$_2$ in causing oxidation of BH$_4$ (14,16,17). These investigators found that ONOO$^-$ dramatically increased vascular superoxide production in vessels from control mice, but not in vessels from eNOS-deficient mice, suggesting that eNOS was the source of superoxide (17).
Cellular BH₄ levels also seem to be dependent on ascorbate. Pretreatment of endothelial cells with ascorbate increases NO• production without affecting NOS expression or L-arginine uptake (18,19). This effect of ascorbate is BH₄ dependent as in the absence of BH₄ it is not observed (18,19). While it is logical to assume that ascorbate may prevent oxidation of BH₄, the precise mechanism whereby ascorbate can enhance cellular levels of BH₄ has not been defined.

In the present study we examined the reaction of ONOO• with BH₄, ascorbate and thiols using electron spin resonance (ESR) and the spin probe 1-hydroxy-3-carboxy-2, 2,5-tetramethylpyrroldidine (CPH). Uncoupling of eNOS by peroxynitrite in cultured bovine aortic endothelial cells (BAECs) was investigated by measuring O₂•– with new cell permeable spin probe 1-hydroxy-3-methoxycarbonyl-2, 2,5,5-tetramethyl-pyrrolidine (CMH) (20), and by measuring nitric oxide using colloidal Fe(DETC)₂, which allows detection and quantification of NO• with high sensitivity and specificity (21,22). We also studied the role of ascorbate on BH₄ oxidation and uncoupling of eNOS and determined whether ascorbate prevents uncoupling of eNOS by scavenging peroxynitrite or if it improves eNOS function by recycling BH₄.

**EXPERIMENTAL PROCEDURES**

**Chemicals and reagents:** (6R)-5,6,7,8-tetrahydrobiopterin and 7,8-dihydro-L-biopterin were purchased from Schircks Laboratories (Switzerland). L-ascorbic acid, glutathione (GSH), cysteine and DMSO were obtained from SIGMA-Aldrich. Peroxynitrite was obtained from Cayman. The ONOO• donor SIN-1 and the cyclic hydroxylamines 1-hydroxy-3-carboxy-2, 2,5-tetramethylpyrrolidine (CPH), 1-hydroxy-3-methoxycarbonyl-2, 2,5,5-tetramethyl-pyrrolidine (CMH) and 2,4-diamino-6-hydroxypyrimidine (DAHP) were purchased from Alexis Corporation (San Diego, USA).
Peroxynitrite concentration was determined spectrophotometrically from its absorbance at 302 nm in 0.1 M NaOH using molar extinction coefficient of 1670. The ESR buffer consisted of sodium phosphate buffer with 2.35 g/L NaH$_2$PO$_4$ + 7.61 g/L Na$_2$HPO$_4$, 0.15 g/L NaCl, 1 g/L Glucose, 0.37 g/L KCl, 0.2 g/L CaCl$_2$ and was treated for 4 hours with 50 g/L chelex 100, a cationic resin, to minimize contamination with transition metals. Krebs-Hepes buffer (KHB) contained 5.786 g/L NaCl, 0.35 g/L KCl, 0.368 g/L CaCl$_2$, 0.296 g/L MgSO$_4$, 2.1 g/L NaHCO$_3$, 0.142 g/L K$_2$HPO$_4$, 5.206 g/L Na-Hepes and 2 g/L D-Glucose.

**Preparation of spin probe and BH$_4$ stock solutions:** Stock solutions of CPH and CMH (10mM) dissolved in 0.9% NaCl containing 1mM diethylenetriamine-pentaacetic acid (DTPA) and purged with argon, were prepared daily and kept under argon on ice. DTPA was used to decrease auto-oxidation of hydroxylamines catalyzed by trace amount of transition metals. CPH and CMH were used in a final concentration of 1mM. BH$_4$ was dissolved in argon purged PBS with DTPA (0.1mM) and kept under argon on ice.

**Cell culture and treatments:** Bovine aortic endothelial cells (BAECs; cell systems, Kirkland, WA) were cultured in Media 199 (M199; Gibco Laboratories) containing 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT) as previously described (23). Confluent BAECs from passage 4 to 7 cultured on 100mm plates were used for ESR experiments. Cell suspensions were used for treatment with ONOO$^-$. For this purpose BAECs were scraped and centrifuged at 1800 rpm for 10 minutes and resuspended in 0.2 ml of ESR buffer. Peroxynitrite (0.27mM) was added to the cell suspension as a bolus and vortexed. ESR measurements of O$_2$•$^-$ were made 3 minutes later. To test if supplementation with BH$_4$ can restore eNOS function after ONOO$^-$, the suspended cells were divided in two Eppendorff tubes and one portion incubated with 20µM BH$_4$ for 4 minutes at room temperature. Some of these cell suspensions were incubated with L-NAME (1 mM) or PEG-SOD (50 U/ml) for 5 minutes and then the spin probe CMH added and vortexed. Superoxide
production was determined by inhibition with 50 U/ml PEG-SOD while superoxide generated by uncoupled eNOS was measured as L-NAME (1mM) inhibited CM* nitroxide formation. In preliminary experiments we confirmed that BH$_4$ (5-10µM) did not interfere with CMH detection of O$_2$•⁻ generated by xanthine and xanthine oxidase.

**Measurements of nitric oxide with Fe(DETC)$_2$:** NO$^*$ production in BAECs has been measured with colloid solution of Fe(DETC)$_2$ as previously described (21,22). Due to its high lipophilicity the formed NO$^*$-Fe(DETC)$_2$ complex is exclusively associated with cell membrane and specifically detects NO$^*$ but not nitrite (21,22). After incubation with Fe(DETC)$_2$ media was aspirated, cells were harvested with a rubber policeman in Krebs-Hepes buffer, resuspended and aspirated into 1ml syringes which were frozen immediately in liquid nitrogen.

**ESR measurements:** Oxidation of the spin probes CPH and CMH by reactive oxygen species (ROS) forms stable nitroxide radicals 3-carboxy-proxyl (CP$^*$) and 3-methoxycarbonyl-proxyl (CM$^*$), which can be assayed by ESR spectroscopy (20,24,25). The amount of nitroxide formed equals the concentration of the reacted oxidant species. Concentration of nitroxides was determined from the ESR amplitude according to calibration curve using standard solutions of the 3-carboxyproxyl radical. ROS formation was measured from the kinetics of nitroxide accumulation by following the ESR amplitude of the low-field component of ESR spectra. The rate of superoxide radical formation was determined by measuring the superoxide dismutase (SOD) inhibited nitroxide generation.

Reactivity of peroxynitrite scavengers with ONOO$^-$ was studied by competition with CPH using both bolus ONOO$^-$ and ONOO$^-$ generated by SIN-1. BH$_4$ and other ONOO$^-$ scavengers compete with CPH to react with ONOO$^-$. The reactivity of each scavenger with bolus ONOO$^-$ was determined using the formula:
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\[
(A_0/A) - 1 = k_{SCAV}/k_{CPH} \times c_{SCAV}/c_{CPH}
\]  
(Formula 1)

Where $A_0$ is the ESR amplitude in absence of ONOO$^-$ scavengers and $A$ the ESR amplitude in presence of ONOO$^-$ scavengers, $k$ is reaction rate constant and $c$ is concentration. With SIN-1 as ONOO$^-$ donor the reactivity of ONOO$^-$ scavengers was calculated using the formula:

\[
(V_0/V) - 1 = k_{SCAV}/k_{CPH} \times c_{SCAV}/c_{CPH}
\]  
(Formula 2)

Where $V_0$ is the rate of nitroxide accumulation in absence of ONOO$^-$ scavengers and $V$ is the rate in presence of ONOO$^-$ scavengers.

ESR samples were placed in 100 $\mu$l capillary and measured at room temperature using a field scan with the following ESR settings: microwave frequency 9.78 GHz, modulation amplitude 2 G, microwave power 10 dB, conversion time 164 msec, time constant 164 msec. Peroxynitrite and ROS production by BAECs were detected by following the low-field peak of the nitroxide ESR spectra using time scans with the following ESR settings: microwave frequency 9.78 GHz, modulation amplitude 2 G, microwave power 10 dB, conversion time 1.3 sec, time constant 5.2 sec.

The intermediate BH$_3^*$ radical was measured by direct ESR spectroscopy without a spin trap. The high-resolution spectrum of the BH$_3^*$ radical was detected and quantified using a microwave frequency 9.78 GHz, modulation amplitude 0.7 G, microwave power 10 dB, conversion time 82 msec and time constant 82 msec.

Frozen probes with NO$^*$-Fe(DETC)$_2$ have a 3-line ESR spectra whose amplitude is proportional to amount of bioactive NO$^*$ produced in cells (21,22,26). Frozen cell samples were measured in a finger Dewar filled with liquid nitrogen at 77K in field scan with following ESR settings: field sweep 160 G, microwave frequency 9.39 GHz, microwave power 20mW, modulation amplitude 3G, conversion time 655 msec, time constant 5242 msec, receiver gain $1 \times 10^4$, number of scans 4.
**Computer simulation of ESR spectra:** Computer simulation of the high-resolution BH$_3^\bullet$ ESR spectra was used for calculation of hyperfine coupling constants. Programs for simulation of ESR spectra and spin trap database are readily available through the Internet (http://epr.niehs.nih.gov/). Details of this computer simulation program have been described elsewhere (27). Hyperfine-coupling constants are expressed as an average of ESR parameters obtained from computer simulation. The ESR spectrum of BH$_3^\bullet$ radical was simulated as a combination of 5 nitrogens with 4 protons with following hyperfine coupling-constants ($a_N$= 8.05 G, $a_N$= 2.31 G, $a_N$= 1.79 G, $a_N$= 1.16 G, $a_N$= 0.93 G, $a_H$=8.41 G, $a_H$=9.50 G, $a_H$=2.50 G, $a_H$=1.06 G).

**Statistical analysis:** Data are presented as mean ± standard error. Analysis with linear regression was done with the software Sigma Plot. For comparison of two groups, a one-tailed t-test was employed using Excel software. Statistical significance was assumed when $p < 0.05$. 

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RESULTS

Reaction of ONOO\textsuperscript{−} with BH\textsubscript{4} and other antioxidants: Previous studies indicated that ONOO\textsuperscript{−} readily oxidizes BH\textsubscript{4} in cultured cells and vessels. We therefore hypothesized that the reactivity of ONOO\textsuperscript{−} with BH\textsubscript{4} would exceed that of ONOO\textsuperscript{−} with other common intracellular antioxidant small molecules. The reactivity of ONOO\textsuperscript{−} with BH\textsubscript{4}, BH\textsubscript{2}, GSH, cysteine, ascorbate and DMSO was studied by examining the competitive reaction between these agents and the hydroxylamine CPH. Boluses of ONOO\textsuperscript{−} (0.27mM) were added to reaction mixtures containing these potential ONOO\textsuperscript{−} scavengers and CPH. In the absence of any scavenger, the reaction of ONOO\textsuperscript{−} with CPH resulted in formation of CP\textsuperscript{•} could be detected as a strong ESR signal (Figure 1A). BH\textsubscript{4} reduced CP\textsuperscript{•} nitroxide generation in a concentration dependent fashion confirming that BH\textsubscript{4} could prevent the reaction of ONOO\textsuperscript{−} with CPH (Figure 1A). In contrast BH\textsubscript{2}, cysteine, GSH, ascorbate and DMSO exhibited substantially less reactivity with peroxynitrite. For comparison of these data, CP\textsuperscript{•} formation by ONOO\textsuperscript{−} was set as 100% and the effectiveness of the various scavengers expressed as a percent of this value. BH\textsubscript{4} strongly inhibited the ESR amplitude by 94%, while ascorbate and thiols inhibited ESR signals to a lesser degree (69% and 63 % respectively). DMSO minimally inhibited the reaction of ONOO\textsuperscript{−} with CPH (24%) (Figure 1B).

By using the ESR amplitudes for the respective reactions as described in the materials and methods and formula 1, the relative reactivity of the antioxidant scavengers with ONOO\textsuperscript{−} was calculated (Figure 2A). As a separate approach to quantify the reactivity of these antioxidants with peroxynitrite, the slopes of the lines presented in figure 2A were compared (figure 2B). According to these data BH\textsubscript{4} reacted with peroxynitrite 10 times faster than ascorbate and 6 times more rapidly than either cysteine or GSH. These data indicate that, in the concentrations employed, neither dihydrobiopterin, ascorbate nor thiols are able to fully protect BH\textsubscript{4} from oxidation by ONOO\textsuperscript{−}.

Reactivity of peroxynitrite scavengers studied with ONOO\textsuperscript{−} donor SIN-1: SIN-1 generates superoxide and nitric oxide resulting in constant production of ONOO\textsuperscript{−}, and therefore serves as a model
for physiological ONOO⁻ production. We therefore studied reactions of various scavengers with ONOO⁻ generated by SIN-1. The rate of ONOO⁻ formation by SIN-1 (5 mM) was measured from the kinetics of CP⁺ nitroxide accumulation by following the ESR amplitude of the low-field component of ESR spectra (Figure 3A, insert). The control sample showed little nitroxide accumulation, while SIN-1 resulted in sharp increase in nitroxide generation (Figure 3A). The accumulation of CP⁺ nitroxide was strongly inhibited by 0.25 mM BH₄ (Figure 3A). BH₄ had the highest reactivity with SIN-1-generated ONOO⁻ followed by cysteine, GSH, ascorbate and DMSO as calculated using formula 2 (Figure 3B). In keeping with our results with bolus addition of ONOO⁻, BH₄ reacted with SIN-1 generated ONOO⁻ 10 times faster than ascorbate and 6 times faster than GSH (Figure 4).

Table 1 provides summary data for experiments with both bolus peroxynitrite and SIN-1. For this analysis, the reactivity of BH₄ with ONOO⁻ (either as a bolus or generated by SIN-1) was set as 100% and compared to reactivities of other antioxidants with ONOO⁻. The reactivity for cysteine, ascorbate and GSH were similar for both systems.

**Formation of BH₃⁺ radical and its reaction with ascorbate and thiols:** The above experiments suggest that ascorbate is only marginally effective in scavenging peroxynitrite. In prior studies, however, it has been reported that ascorbate preserves BH₄ content of purified eNOS permitting full catalytic function of the enzyme. It is also known that ascorbate is incapable of reducing BH₂ back to BH₄ (28). These data suggest that ascorbate may act as a “free radical sink” reducing the intermediate BH₃⁺ radical (29), which may be formed upon the reaction of BH₄ with peroxynitrite. We therefore performed additional experiments to examine interactions between ascorbate, BH₄ and ONOO⁻. While buffer containing BH₄ yielded no ESR signal (Figure 5A), bolus addition of ONOO⁻ (0.27 mM) to BH₄ resulted in formation of a 5-line ESR signal (Figure 5A). Neither decomposed ONOO⁻ nor NaOH (the solvent for ONOO⁻) produced an ESR signal when exposed to BH₄. High-resolution ESR spectra revealed additional hyperfine components
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(Figure 5A). Computer simulation of this high-resolution spectra confirmed assignment of the radial intermediate of BH₄ oxidation by ONOO⁻ as the BH₃⁺ radical (Figure 5A). This computer simulation of the high-resolution spectra of BH₄ and ONOO⁻ was further supported by analysis of ESR spectrum of BH₃⁺ radical in D₂O (data not shown), which was previously reported by Vasquez-Vivar et al. (28).

We next sought to determine if ascorbate or thiols could reduce the BH₃⁺ radical by adding these antioxidants 2-3 seconds after mixing of ONOO⁻ with BH₄. Ascorbate (100 µM) inhibited the BH₃⁺ radical ESR signal by 32% while 1mM ascorbate decreased this signal by 79% (Figure 5B). In contrast, addition of either cysteine or GSH in concentrations of 1 to 10 mM only minimally reduced the ESR signal (Figure 5B). Thus, ascorbate seems to be much more potent than thiol containing compounds in reducing the BH₃⁺ radical.

Recovery of enzymatic activity of uncoupled eNOS in ONOO⁻ treated endothelial cells by BH₄ supplementation: Next we performed experiments to determine if BH₄ was a target of ONOO⁻ oxidation in vivo. To assess function of eNOS in cultured BAECs, the spin probe CMH was used to detect O₂⁻. ROS production by BAECs was measured from the kinetics of CM⁺ nitrooxide accumulation by following the ESR amplitude of the low-field component of ESR spectra (Figure 6A, insert). Untreated cells demonstrated minimal accumulation of nitroxide radical (Figure 6A and 7A). In contrast, cells exposed to peroxynitrite robustly oxidized CMH to CM⁺, and this signal was inhibited by addition of SOD, or by pre-incubation of cells with the NOS inhibitor L-NAME (Figure 6B and 7B). SIN-1 also increased BAEC ROS production, and either L-NAME or SOD inhibited this effect (Figure 6C and 7C). Both bolus ONOO⁻ as well as ONOO⁻ generated by SIN-1 uncoupled eNOS in BAECs in a similar fashion. We have previously shown that O₂⁻ and
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H$_2$O$_2$ react minimally with BH$_4$ (14). In keeping with these previous findings, exposure of BAECs to xanthine (50 µM) and xanthine oxidase (0.5mU/ml), which generates superoxide and hydrogen peroxide, had no effect on subsequent production of O$_2$•$^-$ by endothelial cells (Figure 6D). Nitroxide accumulation was similar to the control cells and did not show inhibition by L-NAME indicating that superoxide did not uncouple eNOS (Figure 6D).

While the effects of ONOO$^-$, SIN-1 and the BH$_4$-synthesis inhibitor DAHP on eNOS function are consistent with depletion of BH$_4$, these agents may have nonspecific effects on endothelial cell NO$^-$ and O$_2$•$^-$ production. We therefore examined if BH$_4$ supplementation was capable of restoring eNOS activity. For this purpose we measured O$_2$•$^-$ by BAECs incubated with exogenous BH$_4$ after the treatment with ONOO$^-$, SIN-1 or DAHP (Figure 7). In preliminary experiments we confirmed that BH$_4$ (5-10µM) did not interfere with CMH detection of O$_2$•$^-$ generated by xanthine and xanthine oxidase.

Treatment of control cells with BH$_4$ increased O$_2$•$^-$ production, and this was unaffected by L-NAME (Figure 7A). In contrast to control cells, supplementation with BH$_4$ concentration-dependently inhibited O$_2$•$^-$ production in cells exposed to bolus ONOO$^-$, as did L-NAME (Figure 7B). A similar effect of BH$_4$ and L-NAME on endothelial O$_2$•$^-$ production was observed in cells that were exposed to either SIN-1 or the inhibitor of BH$_4$ synthesis DAHP (Figure 7C and 7D). Addition of L-NAME to BH$_4$ supplemented cells (Figure 7C and 7D) increased the ESR signal similar to the control cells providing evidence that eNOS function was completely restored.

The activity of eNOS was also determined by measuring NO$^-$ production in BAECs using the NO$^-$-specific spin probe colloid Fe(DETC)$_2$. Because Fe(DETC)$_2$ cannot be used in cell suspensions, and bolus ONOO$^-$ can only be used in cell suspension, we only examined the effect of
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SIN-1-generated ONOO$^-$ on cellular NO$^\cdot$ production. Colloid Fe(DETC)$_2$ in a cell-free sample did not yield an ESR signal. Non-treated control cells demonstrated a strong ESR signal of NO$^\cdot$-Fe(DETC)$_2$ consistent with coupled eNOS function (Figure 8). Treatment of control cells with BH$_4$ slightly reduced NO$^\cdot$ production (Figure 8). We then treated cells with 0.5mM SIN-1 to generate ONOO$^-$ at a rate of 1-1.5µM per minute, levels similar to those observed in pathophysiological conditions (30). This significantly decreased BAEC NO$^\cdot$ production (Figure 8). Following treatment with SIN-1, supplementation with BH$_4$ completely restored NO$^\cdot$ production to values similar to that observed in control cells. Similar to SIN-1, treatment of cells with the BH$_4$ synthesis inhibitor DAHP for 24 hours also decreased NO$^\cdot$ production (Figure 8) and BH$_4$ reversed this effect (Figure 8). Thus, by measuring O$_2^\cdot$ and NO$^\cdot$ from cultured endothelial cells we have shown that ONOO$^-$ derived from SIN-1 and DAHP uncouple eNOS, and that BH$_4$ supplementation corrects this. These data confirm that ONOO$^-$ uncoupled eNOS in BAECs by oxidation of BH$_4$ because supplementation with BH$_4$ after ONOO$^-$ treatment fully restored eNOS function.

Ascorbate prevention of eNOS uncoupling: Our previous data have shown that BH$_4$ administration could recover eNOS function after uncoupling by ONOO$^-$ and also that ascorbate recycles BH$_4$ after its reaction with ONOO$^-$ via reducing the intermediate BH$_3^\cdot$ radical. It was of interest to determine if exogenous BH$_4$ or ascorbate could protect eNOS against ONOO$^-$ in intact endothelial cells. Treatment of control cells with ascorbate did not affect NO$^\cdot$ production (Figure 9). As in figure 8, SIN-1 treatment markedly decreased endothelial cell NO$^\cdot$ production (Figure 9). This effect of SIN-1 was not altered by pretreatment of cells with either ascorbate or BH$_4$ (Figure 9) indicating that saturating cells with ascorbate or BH$_4$ does not prevent eNOS uncoupling when the
cells are subsequently challenged with the ONOO− donor SIN-1. In contrast, co-incubation of cells with BH4 and ascorbate during SIN-1 treatment completely prevented the effect of SIN-1 (Figure 9) on NO• production. The effect of co-incubation with either ascorbate or BH4 alone during SIN-1 treatment was approximately half that of when these agents were used together (Figure 9). These data as well as those presented in figure 5B strongly support the concept that ascorbate protects eNOS from uncoupling by recycling intracellular BH4 (Scheme 2).
DISCUSSION

In the present study, we demonstrated that ONOO$^-$ reacts with BH$_4$ approximately 10 times faster than with ascorbate and 6 times faster than with the thiol-containing compounds glutathione and cysteine. We also showed that this reaction led to formation of the BH$_3$ radical. The BH$_3$ radical was found to have high reactivity with ascorbate, but not with thiols. Finally, we demonstrated that peroxynitrite leads to eNOS uncoupling in cultured endothelial cells and that following exposure of endothelial cells to peroxynitrite, eNOS activity could be fully restored by treatment of the cells with tetrahydrobiopterin. Taken together, these data demonstrate that BH$_4$ is likely a crucial target for ONOO$^-$, and that even in the presence of common cellular antioxidants such as ascorbate and thiols, ONOO$^-$ can lead to BH$_4$ oxidation.

By examining the competition between oxidation of the spin probe CPH and various potential antioxidants, we were able to compare rate constants of ONOO$^-$ reactions with tetrahydrobiopterin, ascorbate and thiols. The rate constants of ONOO$^-$ reactions with ascorbate, cysteine and glutathione have been previously determined to be 236 M$^{-1}$·sec$^{-1}$, 10$^3$ M$^{-1}$·sec$^{-1}$, and 5.8·10$^2$ M$^{-1}$·sec$^{-1}$ (31-33). Table 1 compares ratios of the ONOO$^-$ rate constant with BH$_4$ to the rate constants of other antioxidants. According to these data the rate constant of BH$_4$ is 10-times higher than the rate constant of ascorbate and 6-times higher than the rate constants of thiols. Given our current data, it is possible to estimate the rate constant for the reaction between ONOO$^-$ and BH$_4$ as being 6·10$^3$ M$^{-1}$·sec$^{-1}$.

It has recently been shown that ascorbate treatment increases endothelial cell NO$^*$ synthesis and tetrahydrobiopterin levels (18,19,34), although the mechanism whereby this occurs is not well understood. BH$_2$ is not reduced to BH$_4$ by ascorbate (28), and these prior studies showed that ascorbate did not affect expression of eNOS or GTP-cyclohydrolase, the rate-limiting enzyme for
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BH$_4$ synthesis (18,34). The authors of this prior study indicated that ascorbate stabilized BH$_4$ within the endothelial cell. Our present data provide further insight into this effect of ascorbate. It is unlikely that ascorbate prevents oxidation of BH$_4$ by scavenging ONOO$^-$, as it was found to be 10-fold less reactive with ONOO$^-$ than BH$_4$. Our data indicate that the product of the reaction between ONOO$^-$ and BH$_4$ is the BH$_3$• radical, and that this radical is highly reactive with ascorbate. These data are consistent with recent observation that the BH$_3$• radical is reduced by ascorbate to BH$_4$ with a rate constant of approximately 1.7$\times$10$^5$ M$^{-1}$ s$^{-1}$ (29). Thus, an important mechanism whereby ascorbate stabilizes levels of BH$_4$ seems to involve reduction of the BH$_3$• radical back to BH$_4$, rather than prevention of oxidation of BH$_4$ by oxidants such as ONOO$^-$. According to our data BH$_3$• did not exhibit the same reactivity with thiols, a finding in agreement with previously published data on reactivity of BH$_3$• radical (29). It also seems that ascorbate does not preserve eNOS activity by scavenging ONOO$^-$ or by preventing BH$_4$ from reacting with ONOO$^-$, but that ascorbate improves eNOS function by recycling BH$_4$ (Scheme 2, Fig. 5B). This is supported by our experiments with SIN-1 treated BAECs (Fig. 9), where co-incubation with BH$_4$ and ascorbate fully prevented loss of eNOS function.

Several clinical studies have shown that administration of intraarterial administration of vitamin C can improve endothelium-dependent vasodilatation in the forearms of humans with hypercholesterolemia, diabetes and cigarette smoking (35-37). The effect of vitamin C in these studies has largely been attributed to scavenging of O$_2$•$^-$ (38). While O$_2$•$^-$ scavenging may be a mechanism for improvement in endothelium-dependent vasodilatation in these studies, our current data would indicate that another effect of vitamin C might involve recycling of the BH$_3$• radical to BH$_4$. Our results are consistent with the previously reported data showed that low concentrations of ascorbate stimulate nitric oxide synthase in activated macrophages (39). Thus, recycling of the BH$_3$• radical to BH$_4$ by ascorbate may play an important role in preserving the activity of not only endothelial but also the inducible and neuronal isoforms of nitric oxide synthase.
In keeping with the above findings, we observed that ONOO\(^-\) generated from SIN-1 led to a condition of eNOS uncoupling in cultured cells. This was reflected by a decrease in NO\(^\bullet\) and a concomitant increase in O\(_2\)\(^{--}\) production which could be inhibited by the NOS inhibitor L-NAME.

Recently it has been suggested that ONOO\(^-\) uncouples eNOS by oxidation of the zinc-thiolate complex that comprises the BH\(_4\) binding site. This in turn leads to dissociation of eNOS dimers to monomers (40). In our experiments, however, BH\(_4\) supplementation fully restored NO\(^\bullet\) production in ONOO\(^-\) treated cells, a finding that seems at odds with the concept that the BH\(_4\) binding site is disrupted by ONOO\(^-\). If the zinc-binding site was a primary target for ONOO\(^-\), eNOS would be irreversibly uncoupled and BH\(_4\) supplementation would seem unlikely to restore endothelial cell NO\(^\bullet\) production after exposure to ONOO\(^-\). Our data also demonstrate that the reactivity of BH\(_4\) with ONOO\(^-\) substantially exceeds that of thiols with ONOO\(^-\). Given these considerations, it seems unlikely that ONOO\(^-\) would react with the zinc-thiolate center of eNOS in preference to BH\(_4\). It is possible that both the zinc-thiolate center and BH\(_4\) are targets of oxidation by ONOO\(^-\), particularly when high levels of this oxidant are present, but our data would indicate that BH\(_4\) is preferentially oxidized.

Related to the above discussion, the Previous studies at low temperature SDS-PAGE show that BH\(_4\) markedly stabilized the dimer of eNOS (41,42) by preventing dissociation of the heme (42). Low temperature SDS-PAGE itself, however, affects dimer formation. Therefore, it is unclear whether cellular eNOS in situ exists in the same monomer/dimer forms as is does in gel in vitro. Data obtained with transformed yeast suggest that eNOS does not require BH\(_4\) for dimer formation (7,43). Nevertheless, it has been previously reported that BH\(_4\) increases the critical temperature for dissociation of eNOS dimer from 30–40 °C to 40–50 °C (7). Thus, BH\(_4\) may have some effect on stabilization of the eNOS dimer and oxidation of BH\(_4\) by ONOO\(^-\) is a likely cause of partial dissociation of the eNOS dimer in intact endothelial cells.
BH₄ has been postulated to be deficient in various conditions associated with altered endothelial function (17). Depletion of endothelial BH₄ has been shown to stimulate superoxide production from the isolated eNOS enzyme (44) and form eNOS in intact endothelial cells (45). Supplementation with BH₄ enhances NO• production, improves endothelial-dependent vasodilatation (46), and efficiently couples NADPH oxidation to nitric oxide (NO•) synthesis and inhibits superoxide and hydrogen peroxide formation (8,44). We have shown that oxidation of BH₄ by ONOO− uncouples eNOS, that BH₄ supplementation fully restores eNOS function after uncoupling, providing evidence that BH₄ is a crucial target for peroxynitrite under physiological conditions.

It is now well established that numerous common diseases such as hypercholesterolemia, hypertension, diabetes and heart failure are associated with a loss of NO• production by the endothelium, a condition commonly referred to as endothelial dysfunction (47). In many of these conditions, eNOS uncoupling seems to be present, leading to an increase in endothelial cell O₂•− production and a decrease in NO• production. Our current data, together with other recent publications (14,16,17), strongly suggest that one mechanism leading to eNOS uncoupling is oxidation of BH₄ by ONOO− and similar oxidants. Of note, BH₄ can be administered orally and is effective in treatment of mild phenylketonuria (48). Based on our current findings, it is possible that BH₄ or more likely a combination of BH₄ and vitamin C may prove useful in correcting endothelial dysfunction in these common disorders.

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**FIGURE LEGENDS**

**Figure 1:** Comparison of peroxynitrite scavenging by BH$_4$ and other endogenous antioxidants. (A) ESR spectra of 0.27 mM bolus ONOO$^-$ and CPH in the presence of 0.125 mM, 0.5 mM and 1 mM BH$_4$. BH$_4$ competes with CPH in the reaction with ONOO$^-$ and inhibits CP$^\bullet$ formation by ONOO$^-$ detected by ESR. (B) Inhibition of CP$^\bullet$ nitrooxide formation by ONOO$^-$ in the presence of 0.25 mM peroxynitrite scavengers. Residual ESR signal (%) were compared to ESR amplitude of ONOO$^-$ set as 100%. Compared to the antioxidants BH$_4$ competing with CPH for the reaction with bolus ONOO$^-$ had the highest inhibition on CP$^\bullet$ generation by ONOO$^-$ implying high reactivity with ONOO$^-$. 
Figure 2: Reactivity of potential ONOO⁻ scavengers with bolus ONOO⁻. (A) Inhibition of CP⁺ nitroxide formation by bolus ONOO⁻ in the presence of different peroxynitrite scavengers at varying concentrations: reactivity was calculated using the ESR amplitude (Ao/A)-1 = k_{SCAV}/k_{CPH} x [scav]/[CPH] as described in material and methods. Linear regressions of the data are presented in curves. BH₄-curve has the highest slope implying highest reactivity with ONOO⁻. Slope of BH₄-curve was significantly different from the slopes of the other peroxynitrite scavengers with p < 0.01. (B) Ratio k_{SCAV}/k_{CPH} calculated from the slope of (Ao/A)-1 is shown in (A). Standard errors (SEM) were less than 5%.
Figure 3: Comparison of reactions between various potential ONOO$^-$ scavengers and ONOO$^-$ generated by SIN-1. (A) Insert: ESR spectrum of CPH incubated with 5mM SIN-1. Accumulation of CP$^\cdot$ nitroxide was followed by low-field component of the ESR spectra shown by arrow. Rapid accumulation of CP$^\cdot$-nitroxide in the probe with 5mM SIN-1 was strongly inhibited by 0.25mM BH$_4$. (B) Inhibition of CP$^\cdot$ nitroxide generation by SIN-1 in the presence of peroxynitrite scavengers at varying concentrations: calculation was done using $(Vo/V)-1 = k_{SCAV}/k_{CPH} \times [scav]/[CPH]$ as described in material and methods. Linear regressions of the data are presented in curves. Slope of BH$_4$-curve was significantly different from the other peroxynitrite scavengers with $p < 0.01$. 

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Figure 4: Reactivity of BH₄, thiols and ascorbate with ONOO⁻ generated by SIN-1. Reactivity (kSCAV/kCPH) of various scavengers with ONOO⁻ generated by SIN-1 was calculated from the slopes of (V₀/V)-1 shown in figure 3B. The BH₄-ONOO⁻ reaction yielded the highest slope implying that BH₄ has the highest reactivity with ONOO⁻ of the reductants studied, followed by cysteine, GSH, ascorbate and DMSO. The calculated standard errors (SEM) were less than 7% and BH₄-reactivity was significantly different from the one with other peroxynitrite scavengers (p < 0.01).
Figure 5: Formation of the intermediate BH$_3^*$ radical in the reaction of BH$_4$ with ONOO$^-$. (A) BH$_4$ solution alone (18 mM) has no ESR spectra; ESR signal of 18 mM BH$_4$ plus 0.27mM ONOO$^-$ with a 5-line spectrum; the control samples with 18 mM BH$_4$ plus decomposed ONOO$^-$ and 18 mM BH$_4$ plus NaOH as solvent of ONOO$^-$ had no ESR spectra; high resolution ESR spectrum of 18 mM BH$_4$ plus 0.27mM ONOO$^-$ recorded as described in material and methods. The computer simulation of the high resolution verified that as BH$_3^*$ spectrum. ESR spectrum of BH$_3^*$ radical (E) was simulated as a combination of 5 nitrogens and 4 protons with following hyperfine coupling-constants ($\alpha_N$= 8.05 G, $\alpha_N$= 2.31 G, $\alpha_N$= 1.79 G, $\alpha_N$= 1.16 G, $\alpha_N$= 0.93 G, $\alpha_H$=8.41 G, $\alpha_H$=9.50 G, $\alpha_H$=2.50 G, $\alpha_H$=1.06 G). (B) Reactivity of BH$_3^*$ radical with the antioxidants ascorbate and thiols: the effect of ascorbate or thiols at different concentrations on the ESR signal of BH$_3^*$ radical is presented in percentage of ESR amplitude. The antioxidants were added few seconds after the reaction of BH$_4$ with bolus ONOO$^-$. Data confirm high reactivity of BH$_3^*$ radical with ascorbate but not with thiols.
Figure 6: Superoxide production by endothelial cells following exposure to ONOO\textsuperscript{−} or SIN-1. ROS formation was measured in BAECs after treatment with bolus ONOO\textsuperscript{−}, ONOO\textsuperscript{−} donor SIN-1 or with superoxide generated by xanthine and xanthine oxidase as accumulation of CM\textsuperscript{•} nitroxide, which was followed by low-field component of the ESR spectra shown by arrow in insert (A). Superoxide production was determined by inhibition with 50 U/ml PEG-SOD while superoxide generated by uncoupled eNOS was measured as L-NAME (1mM) inhibited CM\textsuperscript{•} nitroxide formation. (A) CM\textsuperscript{•} accumulation in non-treated control cells. The inhibition of coupled eNOS with L-NAME increased amount of detected superoxide. (B, C) ROS production in BAECs treated by bolus 0.27mM ONOO\textsuperscript{−} or 0.5 mM SIN-1. The inhibition of uncoupled eNOS with L-NAME decreased amount of detected superoxide. (D) ROS production in BAECs treated by 50µM xanthine and 0.5mU/ml xanthine oxidase.
Figure 7: Effect of BH₄ supplementation on superoxide production by uncoupled eNOS in BAECs treated with ONOO⁻, SIN-1 and DAHP. (A) CM• nitroxide accumulation of control cells before and after BH₄ (20µM) supplementation and the inhibition by L-NAME (1mM). (B) ROS production by endothelial cells treated with 0.27mM ONOO⁻ and inhibition of CM• accumulation by L-NAME. To test concentration dependent effect of BH₄ we compared supplementations with 10 µM and 20 µM BH₄ after BAECs were treated with bolus ONOO⁻ (B). (C) CM• accumulation in BAECs treated with 0.5mM SIN-1. L-NAME strongly inhibited by superoxide production in SIN-1 treated cells, while supplementation with BH₄ abolished effect of L-NAME. (D) CM• accumulation in BAECs treated with an inhibitor of BH₄ synthesis DAHP (5mM). Supplementation with BH₄ (20µM) restored eNOS function.
Figure 8: Effect of ONOO$^-$ donor SIN-1, DAHP and BH$_4$ supplementation on NO$^*$ production in endothelial cells. NO$^*$ production in cultured BAECs was detected as NO$^*$-Fe(DETC)$_2$ as described in material and methods. Production of NO$^*$ in BAECs was measured in untreated control cells, control cells supplemented with 20µM BH$_4$, in 0.5mM SIN-1 treated cells, BH$_4$ supplemented cells after SIN-1 treatment, 5mM DAHP treated cells and BH$_4$ supplemented cells after DAHP treatment. Amplitude of ESR signal corresponds to bioavailable NO$^*$ in cells and is shown in % compared to signal of control cells set as 100%.
Figure 9: Effect of ascorbate on NO\(^{\bullet}\) production in ONOO\(^{-}\) (SIN-1) treated BAECs. Production of NO\(^{\bullet}\) in BAECs was measured as NO\(^{\bullet}\)-Fe(DETC)\(_2\) in untreated control cells, 100 µM ascorbate treated cells, 0.5mM SIN-1 treated cells, cells incubated with ascorbate before treatment with 0.5mM SIN-1, cells incubated with both ascorbate plus SIN-1, cells incubated with BH\(_4\) before treatment with 0.5mM SIN-1, cells simultaneously incubated with BH\(_4\) plus SIN-1 and in cells co-incubated with both BH\(_4\) and ascorbate during SIN-1 treatment. Amplitude of ESR signal (%) was compared to ESR signal of control cells set as 100%.
Table 1. Reactivity of peroxynitrite scavengers with bolus ONOO$^-$ and SIN-1 generated ONOO$^-$.

Reactivity of BH$_4$ with bolus ONOO$^-$ or ONOO$^-$ generated by SIN-1 was set 100%± standard errors (SEM) in % and compared to the reactivity of the other antioxidants with ONOO$^-$. The reactivity of ascorbate and GSH were in the same range in both systems with some variation for cysteine and DMSO.

<table>
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<tr>
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<th>ONOO$^-$</th>
<th>SIN-1</th>
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<tbody>
<tr>
<td>BH$_4$</td>
<td>100±1.4 %</td>
<td>100±3.1 %</td>
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<tr>
<td>BH$_2$</td>
<td>30.8±0.7 %</td>
<td>—</td>
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<tr>
<td>Cysteine</td>
<td>16.5±0.6 %</td>
<td>27.4±0.9 %</td>
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<tr>
<td>GSH</td>
<td>15.0±0.7 %</td>
<td>15.4±0.8 %</td>
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<tr>
<td>Ascorbate</td>
<td>10.4±0.1 %</td>
<td>11.4±0.5 %</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.2±0.01 %</td>
<td>1.6±0.1 %</td>
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Scheme 1. Chemical structure of (6R)-5,6,7,8-tetrahydrobiopterin (BH₄)

Scheme 2. Mechanism of the reaction between ONOO⁻ and BH₄ and the role of ascorbate (AH⁻).

ONOO⁻ oxidizes BH₄ to the intermediate BH₃⁺ radical, which can decay to BH₂ or can be converted back to BH₄ by ascorbate.
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Uncoupling of eNOS by ONOO


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