Endothelial Nitric Oxide Synthase–Derived Nitric Oxide Prevents Dihydrofolate Reductase Degradation via Promoting S-Nitrosylation

Zhejun Cai, Qiulun Lu, Ye Ding, Qilong Wang, Lei Xiao, Ping Song, Ming-Hui Zou

Objective—Dihydrofolate reductase (DHFR) is a key protein involved in tetrahydrobiopterin (BH₄) regeneration from 7,8-dihydrobiopterin (BH₂). Dysfunctional DHFR may induce endothelial nitric oxide (NO) synthase (eNOS) uncoupling resulting in enzyme production of superoxide anions instead of NO. The mechanism by which DHFR is regulated is unknown. Here, we investigate whether eNOS-derived NO maintains DHFR stability.

Approach and Results—DHFR activity, BH₄ content, eNOS activity, and S-nitrosylation were assessed in human umbilical vein endothelial cells and in aortas isolated from wild-type and eNOS knockout mice. In human umbilical vein endothelial cells, depletion of intracellular NO by transfection with eNOS-specific siRNA or by the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO)—both of which had no effect on DHFR mRNA levels—markedly reduced DHFR protein levels in parallel with increased DHFR polyubiquitination. Supplementation of S-nitroso-

Conclusions—We conclude that S-nitrosylation of DHFR at cysteine 7 by eNOS-derived NO is crucial for DHFR stability. We also conclude that NO-induced stabilization of DHFR prevents eNOS uncoupling via regeneration of BH₄, an essential eNOS cofactor. (Arterioscler Thromb Vasc Biol. 2015;35:2366-2373. DOI: 10.1161/ATVBAHA.115.305796.)

Key Words: human umbilical vein endothelial cells ■ nitric oxide ■ Nos3 protein, mouse ■ sapropterin ■ tetrahydrofolate dehydrogenase

Nitric oxide (NO), an essential molecule in maintaining cardiovascular health, is derived from \( \text{L-arginine} \) in an oxidizing reaction catalyzed by NO synthase (NOS). Three distinct isoforms of NOS have been identified in mammalian cells: neuronal NOS, inducible NOS, and endothelial NOS (eNOS). The eNOS isoform is a major source of NO produced by endothelial cells, and an overwhelming amount of evidence indicates that dysfunctional eNOS is associated with the pathogenesis of various vascular diseases, including atherosclerosis and hypertension. Under such conditions, endothelial NO bioactivity is diminished and oxidative stress increases, resulting in endothelial dysfunction.

See accompanying editorial on page 2261

The enzymatic function of eNOS is tightly regulated by various factors. One cofactor, tetrahydrobiopterin (BH₄), helps to maintain enzyme coupling. When oxidized or reacted with peroxynitrite, BH₄ forms 7,8-dihydrobiopterin (BH₂) and trihydrobiopterin radical accordingly, which inactivates NOS function by competing with BH₄ for eNOS binding; this ultimately causes NOS uncoupling. Under such condition, the enzyme is converted from an NO-producing enzyme to a molecule that generates superoxide anions.

Two key enzymes regulate the concentration of BH₄. GTP cyclohydrolase I (GTPCH) controls de novo biosynthesis from GTP, and dihydrofolate reductase (DHFR) is the key enzyme responsible for salvage of BH₄ from BH₂ at the expense of nicotinamide adenine dinucleotide phosphate oxidase. Recent studies indicate that angiotensin II downregulates DHFR expression, decreases BH₄ levels, and increases eNOS uncoupling in endothelial cells through oxidative stress. In addition, DHFR inhibition or knockdown diminishes the BH₄:BH₂ ratio and exacerbates NOS uncoupling. In fact, BH₂-deficient (hph-1) mice treated with methotrexate to inhibit BH₄ recycling by DHFR displayed strikingly elevated BH₂ levels and decreased BH₄:BH₂ ratio in the aortas.© 2015 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.115.305796
### Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>BH&lt;sub&gt;4&lt;/sub&gt;</td>
<td>7,8-dihydrobiopterin</td>
</tr>
<tr>
<td>BH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>tetrahydrobiopterin</td>
</tr>
<tr>
<td>DHFR</td>
<td>dihydrofolate reductase</td>
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<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<tr>
<td>GSNO</td>
<td>S-nitroso-l-glutathione</td>
</tr>
<tr>
<td>GTPCH</td>
<td>GTP cyclohydrolase I</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>PTIO</td>
<td>2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide</td>
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Despite results from previous studies suggesting that GTPCH and DHFR mediate eNOS function, it is unknown whether eNOS feedback regulates these 2 enzymes.

In addition to vasodilation, NO is also involved in various signaling pathways. In these pathways, NO targets specific cysteine residues, resulting in covalent incorporation of NO into thiol groups to form S-nitrosothiol, termed S-nitrosylation. We previously reported that NO can activate GTPCH activity through S-nitrosylation in endothelial cells. However, it is unknown whether eNOS-derived NO has the potential to S-nitrosylate DHFR to regulate its function.

DHFR expression is regulated by various mechanisms, including transcription and degradation, which are mediated via the ubiquitin–proteasome pathway. Previous reports suggest that certain post-translational modifications may regulate protein ubiquitination and function. S-nitrosylation in particular may inhibit degradation of certain proteins, such as Bcl-2 (B-cell lymphoma 2) and TRIM72 (tripartite motif-containing protein 72) and promote degradation of other proteins, such as Parkin. In this study, we hypothesized that NO generated by eNOS stabilizes DHFR via S-nitrosylation, which regenerates BH<sub>4</sub> to maintain the eNOS-coupling status.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

### Results

**eNOS Silencing Reduces DHFR Protein Expression in Human Umbilical Vein Endothelial Cells**

Confluent human umbilical vein endothelial cells (HUVECs) express high levels of eNOS, GTPCH, and DHFR (Figure 1A). To determine whether eNOS expression alters GTPCH and DHFR levels, confluent HUVECs were transfected with scramble siRNA or eNOS-specific siRNA. eNOS-specific siRNA markedly reduced DHFR protein levels compared with those transfected with control siRNA, 48 hours after transfection (Figure 1A), indicating that eNOS-specific siRNA targets only eNOS. Neither control siRNA nor eNOS-specific siRNA affected the levels of GTPCH (Figure 1A). Interestingly, DHFR protein expression in eNOS-silenced HUVECs was markedly lower than that in HUVECs transfected with control siRNA (Figure 1A); however, eNOS silencing did not significantly alter DHFR mRNA levels (Figure 1B).

We also tested the effect of eNOS inhibition by N-nitro-L-arginine methyl ester (L-NNAME) on DHFR and GTPCH expression. Similar to the results of eNOS siRNA silencing, L-NNAME reduced DHFR expression from the 1 mmol/L to 2 mmol/L but had no effect on GTPCH expression (Figure 1 in the online-only Data Supplement).

**eNOS-Derived NO Prevents DHFR Reduction in HUVECs**

The major function of eNOS is to generate NO. Next, we investigated whether reduced NO release in eNOS-silenced HUVECs results in reduced expression of DHFR. To this end, HUVECs were exposed to 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), a known NO scavenger. As shown in Figure 1C, treatment of HUVECs with PTIO for 24 hours dose dependently reduced DHFR protein levels from 150 to 300 μmol/L. Exposure of HUVECs to PTIO (150 μmol/L) for 6 and 12 hours had marginal effects on DHFR expression. Prolonged exposure of HUVECs to PTIO for 24 to 48 hours significantly reduced DHFR (Figure 1C). PTIO did not alter DHFR mRNA levels (Figure 1D). In addition, PTIO incubation did not alter GTPCH expression at a maximum dose of 300 μmol/L for 48 hours (Figure 1C).

Because both eNOS silencing and NO scavenging via PTIO lowered DHFR levels in HUVECs, we reasoned that exposure of HUVECs to a NO donor would ablate DHFR reduction in eNOS-silenced HUVECs. As depicted in Figure 1E and 1F, exposure of HUVECs to S-nitroso-l-glutathione (GSNO), a NO donor, reversed DHFR reduction induced by eNOS siRNA transfection or PTIO incubation. These results indicate that eNOS-derived NO is essential for maintaining DHFR expression without affecting mRNA levels.

**Peroxynitrite Does Not Affect DHFR Expression in HUVECs**

Superoxide anions could deplete NO to generate peroxynitrite (ONOO⁻), which is crucial in the pathogenesis in hypertension and endothelial dysfunction. We further tested whether ONOO⁻ may affect DHFR in HUVECs. As shown in Figure 2I in the online-only Data Supplement, addition of ONOO⁻ ranging from 50 to 500 μmol/L induced protein tyrosine nitration in a dose-dependent manner. GTPCH expression decreased as the increased dose of ONOO⁻, which is consistent with our previous report. However, DHFR expression remained unchanged, indicating that ONOO⁻ has no effect on DHFR expression.

**NO Stabilizes the DHFR Protein in HUVECs**

Because NO depletion in HUVECs only suppressed DHFR protein expression without altering mRNA levels, we investigated whether NO affects the stability of the DHFR protein. To this end, protein stability (half-life) was measured in the presence of cycloheximide, an inhibitor of de novo protein synthesis. As depicted in Figure 2A, in the absence of cycloheximide, the DHFR protein decreased by ≈50% after 4 hours and continued to decline after 8 hours. As depicted in Figure 2A, GSNO treatment significantly inhibited DHFR reduction in the presence of cycloheximide, and the DHFR half-life was increased to 7.86 hours.
NO Depletion Promotes DHFR Degradation via the Ubiquitin–Proteasome System in HUVECs

The ubiquitin–proteasome system is important for intracellular protein degradation. It has been reported that DHFR can be ubiquitinated and degraded by the proteasome. To examine whether NO depletion caused by eNOS silencing or PTIO leads to DHFR reduction via ubiquitin–proteasome degradation, eNOS siRNA–treated or PTIO-treated HUVECs were coincubated with MG132, a potent 26S proteasome inhibitor. As depicted in Figure 2B and 2C, MG132 ablated DHFR reduction caused by eNOS silencing or PTIO. In parallel, PTIO increased the detection of ubiquitinated DHFR.
(Figure 2D). GSNO pretreatment abolished PTIO-enhanced DHFR ubiquitination. These data suggest that NO via the ubiquitin–proteasome system promotes DHFR ubiquitination and 26S proteasome–mediated degradation.

**NO Depletion Reduces DHFR Activity and BH₄ Levels in HUVECs**

DHFR is the key enzyme responsible for salvation formation of BH₄.⁹ Therefore, we determined whether NO depletion altered the function of DHFR in HUVECs. In parallel with decreased protein levels, DHFR activity and intracellular BH₄ were markedly suppressed by eNOS silencing. These effects were restored by MG132 or GSNO treatment (Figure IIIA–IIID in the online-only Data Supplement). Like the effects of eNOS silencing, PTIO also led to a reduction in DHFR activity and BH₄ levels, all of which were prevented by addition of MG132 or GSNO (Figure IIIE–IIIH in the online-only Data Supplement).

**NO S-Nitrosylates DHFR at Cysteine 7 in HUVECs**

Next, we sought to explore how NO regulates DHFR. Since NO leads to S-nitrosylation of certain proteins,¹⁸,²⁰ we tested whether NO could also S-nitrosylate DHFR. As shown in Figure 3A, GSNO markedly increased DHFR S-nitrosylation, whereas PTIO significantly reduced S-nitrosylation.

There is only 1 cysteine residue (C7) in human DHFR, which is conserved among species (Figure 3B), suggesting that it is the residue to be S-nitrosylated. To confirm cysteine 7 is the target for S-nitrosylation, we generated a DHFR mutant, in which cysteine 7 of DHFR was replaced with serine (C7S). Both wild-type (WT) DHFR and the C7S mutant were transfected into HUVECs. After transfection, the cells were treated with GSNO for 6 hours. As expected, the C7S mutation in DHFR blocked basal- and GSNO-induced S-nitrosylation of DHFR (Figure 3C).

**C7 S-Nitrosylation Stabilizes DHFR in HUVECs**

S-nitrosylation of certain proteins may influence their stability.¹⁹,²⁴ We explored whether C7 S-nitrosylation of DHFR affects its stability. WT or C7S mutant DHFR plasmids were transfected into HUVECs. The C7S mutation of DHFR shortened the half-life of the protein compared with the WT (Figure 4A). GSNO significantly increased the half-life of WT DHFR, but the C7S mutation blocked this effect in HUVECs (Figure 4A). These data suggest that S-nitrosylation of DHFR at C7 stabilizes the protein.

**S-Nitrosylation of DHFR Prevents It From Ubiquitination and Degradation**

Because the ubiquitin–proteasome system is involved in NO depletion–induced DHFR degradation, we next determined whether S-nitrosylation of DHFR initiates ubiquitination and degradation of DHFR. As expected, in parallel with increased S-nitrosylation (Figure 4B), GSNO supplementation markedly lowered PTIO-enhanced DHFR ubiquitination in HUVECs (Figure 4C).

NO directly regulates proteasome activity.²³ To exclude the possibility that NO-suppressed DHFR degradation occurs via suppressed proteasome activity, HUVECs were treated with dithiothreitol, a known inhibitor of S-nitrosylation,²⁵ in addition to GSNO and PTIO. As shown in Figure 4E, PTIO increased proteasome activity, which was reversed by addition of GSNO. Although supplementation with dithiothreitol blocked the effects of GSNO on DHFR ubiquitination and degradation (Figure 4C and 4D), it had no effect on proteasome activity (Figure 4E).

**S-Nitrosylation of DHFR Does Not Affect Its Activity In Vitro**

Next, we tested whether S-nitrosylation of DHFR at C7 affects its activity. Recombinant His-tagged WT and C7S DHFR proteins were successfully generated and incubated with GSNO to induce S-nitrosylation. As expected, GSNO increased S-nitrosylation of WT DHFR but had no effect on S-nitrosylation in C7S DHFR mutation in vitro (Figure 4A in the online-only Data Supplement). DHFR activity was further assessed. As shown in Figure 4B in the online-only Data Supplement, the in vitro assay showed no difference between the WT and C7S DHFR activities, and GSNO incubation did not affect their activities.

**NO Depletion Suppresses Aortic Endothelial DHFR Expression and BH₄ Content via Proteasomal Degradation Ex Vivo**

The effects of NO and proteasome degradation on endothelial DHFR in aortas were further tested in ex vivo system. Aortic segments isolated from 8-week-old mice were incubated with PTIO for 24 hours. As shown in Figure 5A to 5C, PTIO...
markedly reduced DHFR expression in endothelium, as determined by immunofluorescence staining and Western blots. MG132 treatment prevented the effects of PTIO on DHFR (Figure 5A–C). As expected, the endothelial GTPCH expression was not altered by PTIO incubation (Figure 5D and 5E).

We next assayed the BH$_4$ and total biopterin levels in ex vivo cultured aortas. Similar to the effect on DHFR protein expression, PTIO significantly reduced BH$_4$ levels, which was restored by MG132 (Figure 5F).

eNOS Deficiency Reduces Endothelial DHFR Expression and BH$_4$ Content via Proteasomal Degradation In Vivo
Because the above data show that NO prevents endothelial DHFR degradation both in HUVECs and ex vivo aortas, we reasoned that eNOS deficiency may lead to reduced endothelial DHFR expression and BH$_4$ content. Indeed, immunofluorescence staining revealed reduced endothelial DHFR expression in aorta from eNOS$^{-/-}$ mice compared with WT mice (Figure 6A and 6B). In contrast, the expression of GTPCH was comparable between WT and eNOS$^{-/-}$ mice (Figure 6C and 6D).

Next, we determined whether proteasome inhibition increased the levels of DHFR in the aortas of eNOS$^{-/-}$ mice. To test this, both WT and eNOS$^{-/-}$ mice received intraperitoneal injection of MG132. As depicted in Figure 6A and 6B, MG132 increased the levels of DHFR in endothelial cells. Finally, we detected BH$_4$ and total biopterin levels in aortas. As expected, aortas from eNOS$^{-/-}$ mice showed reduced levels of BH$_4$ compared with WT mice, which was reversed by MG132 intraperitoneal injection (Figure 6E). These data further support that NO prevents endothelial DHFR degradation through the proteasome system both in vitro and in vivo.

Discussion
In this study, we demonstrate for the first time that NO derived from eNOS regulates DHFR expression and function. The major finding in this study is that NO generated by eNOS promotes DHFR S-nitrosylation at C7. Furthermore, we found that S-nitrosylation of DHFR inhibits DHFR ubiquitination and proteasome degradation. Stimuli that deplete intracellular NO lead to the reduction of DHFR S-nitrosylation and result in its degradation through the ubiquitin–proteasome system. The NO donor GSNO efficiently reversed the effects. Consistently, DHFR is markedly lower in the endothelium from eNOS$^{-/-}$ mice when compared with those in WT mice. Finally, proteasome inhibition with MG132 increased DHFR expression in the endothelium of eNOS$^{-/-}$ mice. Taken together, we conclude that NO derived from eNOS is a key factor in maintaining DHFR stability, BH$_4$ contents, and eNOS uncoupling.

It has been well documented that DHFR and GTPCH regulate eNOS coupling via maintaining BH$_4$ content. We show here that eNOS deficiency reduces DHFR but not GTPCH expression both in vivo and in vitro. The major
function of eNOS is to generate NO, and studies reveal that NO participates in various signaling pathways and exerts multibiological functions.27 Our data show that NO also regulates DHFR expression. We found that depletion of NO by eNOS deficiency or PTIO reduced DHFR protein levels, paralleled by reduced DHFR activity and intracellular BH4 content but not by a reduction in mRNA levels. This suggests post-transcriptional regulation of DHFR is responsible for the reduction. Indeed, we found PTIO increased DHFR ubiquitination. The ability of the 26S proteasome inhibitor MG132 to suppress DHFR reduction caused by NO depletion strongly supports the role of the proteasome pathway in DHFR degradation.

NO reacts with cysteine residues to form S-nitrosothiol, and the S-nitrosylated proteins have been increasingly recognized as important determinants of many biochemical processes.28 Our laboratory previously reported that NO is able to S-nitrosylate GTPCH, the key enzyme in de novo BH4 synthesis pathway.15 However, whether NO can also S-nitrosylate DHFR and how it affects the protein remains unknown. Here, we found that NO donor GSNO increased DHFR S-nitrosylation, whereas NO scavenger PTIO decreased its S-nitrosylation. We further identified that C7 of DHFR is the site of S-nitrosylation, which leads to increased stability. Addition of the NO donor GSNO increased DHFR C7 S-nitrosylation and suppressed DHFR reduction in the presence of cycloheximide. However, mutation of this cysteine residue blocked the effect. These data support the role of C7 S-nitrosylation on DHFR degradation process.

S-nitrosylation of proteins is reported to regulate their ubiquitination and stability.26,29 Our results show that S-nitrosylation of DHFR prevents it from ubiquitination, thereby reducing degradation. The mechanism by which S-nitrosylation prevents DHFR ubiquitination is unclear but may attribute to the conformational change of the protein, which prevents recognition and subsequent attachment of ubiquitin by the enzyme ubiquitin ligases.

This study demonstrates that NO could stabilize DHFR via S-nitrosylation and therefore help to maintain eNOS-coupling status. However, it is also reported that overproduction of NO could also S-nitrosylate eNOS and suppress its function.30 These data suggest that 2 mechanisms of NO S-nitrosylation may work together to maintain eNOS in normal function.

This study used PTIO as an NO scavenger. However, it is reported that PTIO reacts with NO and generates NO2 radicals,31 which could then lead to tyrosine nitration of proteins. Despite this side effect of PTIO, our results already showed that tyrosine nitration caused by ONOO- had no effect on DHFR, which indicates that the effect of PTIO on DHFR was not because of tyrosine nitration.

BH4 plays an important role in eNOS coupling, which is crucial in maintaining endothelial function.32 In fact, supplementation of BH4 would seem to be a promising strategy for improving the vascular status in such diseases. Unfortunately,
application of this strategy to improve endothelial function in vascular disease has resulted in less than satisfactory results.33,34 Studies show that treatment of human endothelial cells with BH4 may only transiently increase intracellular BH4, which is quickly oxidized, causing the accumulation of BH2. This form is inactive as a NOS cofactor and may compete with BH4 for NOS binding and increase eNOS uncoupling.13,34 This may limit the benefits of BH4 therapies but suggest that DHFR controlling the salvage pathway of BH4 synthesis might be more crucial in balancing intracellular BH4:BH2 ratio than we originally thought.

In summary, this study provides evidence to suggest that eNOS-derived NO is critical for maintaining DHFR expression in endothelial cells. Stimuli causing NO depletion induces DHFR downregulation through ubiquitin–proteasome–dependent degradation. This is mediated by reduced S-nitrosylation DHFR at C7, which leads to its instability. This signaling cascade may represent a common mechanism that eNOS regulates its homeostasis by maintaining DHFR S-nitrosylation.

**Sources of Funding**
This study was supported by grants (HL079584, HL080499, HL089920, HL105157, HL110488, HL128014, and AG047776) to M.H. Zou from the National Institutes of Health.

**Disclosures**
None.

**References**


**Significance**

Dihydrofolate reductase (DHFR) is a key protein involved in BH4 regeneration from BH2. BH4 is a cofactor that maintains proper function of endothelial nitric oxide synthase and endothelial function. Dysfunctional DHFR is reported to uncouple endothelial NO synthase resulting in enzyme production of superoxide anions rather than NO. Here, we demonstrate that endothelial NO synthase may also regulate DHFR by generating NO leading to DHFR S-nitrosylation. S-nitrosylation stabilizes DHFR and thus maintains BH4 levels in endothelial cells. We also show that stimuli, which deplete NO, induce endothelial DHFR both in vitro and in vivo via ubiquitin–proteasome degradation. These findings highlight the role of NO in maintaining DHFR stability and activity. They also suggest that endothelial NO synthase has the ability to maintain its coupling status through NO/DHFR S-nitrosylation feedback.
Endothelial Nitric Oxide Synthase–Derived Nitric Oxide Prevents Dihydrofolate Reductase Degradation via Promoting S-Nitrosylation
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Arterioscler Thromb Vasc Biol. 2015;35:2366-2373; originally published online September 17, 2015;
doi: 10.1161/ATVBAHA.115.305796

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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**Materials and Methods**

**Chemicals and antibodies**

2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), S-Nitroso-L-glutathione (GSNO), and MG132 were purchased from Cayman Chem (Ann Arbor, MI). Dithiothreitol (DTT), cycloheximide (CHX), and Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) were from Sigma-Aldrich (St. Louis, MO). Peroxynitrite and its degraded form were purchased from Merck (Temecula, CA). Protein A-Sepharose CL-4B beads were from GE healthcare (Pittsburgh, PA). Antibodies against DHFR, β-actin, ubiquitin, and eNOS were from Santa Cruz Biotechnology (Santa Cruz, CA), GTPCH was from Sigma-Aldrich, Myc was from Cell Signaling Technology (Beverly, MA), CD31 was from BD Pharmingen (San Jose, CA).

**Animals**

Eight-week-old C57BL/6J and eNOS⁻/⁻ mice were obtained from the Jackson Laboratory (Bar Harbor, Me). Mice were housed in temperature-controlled cages with a 12-hour light/dark cycle and given free access to water and food. The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center.

**Cell culture**

Human umbilical vein endothelial cells were cultured in endothelial basal medium (Lonza, Allendale, NJ) supplemented with EGM-2 Bullet Kit (Lonza), 5% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), 100 units/ml penicillin, 100µg/ml streptomycin, and 2mM L-glutamine in a humidified atmosphere of 5% CO₂ at 37 °C. Cells were treated with indicated chemicals for 24h before harvested for subsequent analysis. For ubiquitination and S-nitrosylation experiments, cells were collected 6h after indicated treatment. Cells were used within 8th generation.
Plasmids and Transfection
The Myc-DHFR and control pCMV6-entry plasmid were purchased from Origene (Rockville, MD). DHFR C7S mutant (Myc tagged) was generated using the QuickChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer’s instructions. The mutation was verified by DNA sequencing. For E. coli (BL21) protein expression, DHFR and C7S mutant were inserted into pET28a (Amersham Pharmacia Biotech).

Cellular small-interfering RNA and plasmids transfection
HUVECs were transfected according with either control small-interfering RNA (siRNA) or eNOS siRNA duplex (Santa Cruz Biotechnology) with RNAiMax (Life Technology, Grand Island, NY). The plasmids were transfected into cells using P5 Primary Cell 4D-Nucleofector® X Kit L electroporation kit from Lonza (Walkersville, MD), following manufacturer’s instruction.

Expression and purification of recombinant His-DHFR in bacteria
His-DHFR or C7S-DHFR were expressed in the E. coli BL21. The cells were resuspended in lysis buffer [100 mM Tris-HCl pH 8.5, 100 mM NaCl, 10% glycerol, 1% Triton X-100, and protease inhibitors], then lysed by brief sonication. The His- tagged proteins were produced according to the manufacturer’s manual (The QIAexpressionist, Qiagen).

Immunoprecipitation and immunoblotting
Cells were lysed with RIPA buffer from Santa Cruz Biotechnology. Lysates were centrifuged at 10,000 g for 10 min at 4 ºC. Cleared lysates were incubated with the indicated antibodies overnight and 1 h with protein A-Sepharose beads. The pellets were then washed five times with ice-cold lysis buffer and re-suspended in SDS sample buffer. Eluted immunoprecipitates or whole cell lysates were separated by SDS-PAGE and analyzed by immunoblotting.
**Realtime PCR for DHFR**
RNA was isolated from the treated HUVECs with the RNeasy Mini Kit (Qiagen, Valencia, Calif), and then reverse-transcripted into cDNA by iScript cDNA synthesis kit (Bio-rad, Hercules, CA). Forward: 5’ – TCGCTAAACTGCATCGTCGCTGTGTC- 3’ and Reverse: 5’ – TGGAGGTTCCTTGAGTTCTCTGCTGA- 3’ were used for the following Realtime PCR analysis as described previously¹.

**Determination of BH₄ and Total Biopterins**
The levels of BH₄ and total biopterins were determined via differential oxidation followed by high-performance liquid chromatography quantification, as described previously²,³.

**Detection of protein S-nitrosylation with the biotin-switch**
S-nitrosylated DHFR was determined by using the kits from Cayman Chemicals, according to manufacturer’s instruction and as described previously³.

**DHFR activity assay**
Cells were harvested and DHFR activity was measured using the DHFR assay kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s instructions and previous report⁴.

**Determination of 26S proteasome activity**
Cells were incubated with 1μM of Me4BodipyFL-Ahx₃Leu₃VS (BostonBiochem, Boston, MA) for 30min. After washing with PBS for 3 times, the 26S proteasome Cleavage activity was monitored by Infinite M1000 plate reader (Tecan, San Jose, CA) at 515/519 nm. The results were calibrated by the protein concentration.
**Ex vivo aortic culture**

The thoracic aortas were collected and subsequently cultured in endothelial basal medium (Lonza) supplemented with EGM-2 Bullet Kit (Lonza), 100 units/ml penicillin, 100µg/ml streptomycin, and 2mM L-glutamine in a humidified atmosphere of 5% CO₂ at 37 °C with indicated treatment for 24h.

**Treatment with MG132 in mice**

Eight-week-old mice were subjected into four groups: wild type (WT), WT+MG132, eNOS⁻/⁻, eNOS⁻/⁻+MG132, n=5 for each group. For mice treated with MG132 treatment, 5mg/kg/d of MG132 was given intraperitoneally for 3 days, while the WT and eNOS⁻/⁻ groups received ip injection of identical amount of vehicle (DMSO dissolved in PBS) for 3 days.

**Immunofluorescence**

The thoracic aorta cryosections were fixed in cold acetone, and rinsed by PBS subsequently. Samples were blocked with protein block solution (Protein Block), and then incubated with primary antibody (anti-CD31 from BD; anti- DHFR from Stanta Cruz; anti- GTPCH from Sigma-Aldrich) overnight at 4°C. Slides were rinsed and incubated with secondary antibody Alexa 555 goat anti-rabbit and Alexa 488 goat anti-rat for 1h, and then washed and observed under fluorescent microscope. Semiquantitative analysis of tissue immunoreactivity was done by 4 observers blinded to the identity of the samples using an arbitrary grading system from score 1 to 4 (score 1: 0-25% positive staining in intima; score 2: 26-50% positive staining in intima; score 3: 51-75% positive staining in intima; score 4: 76-100% positive staining in intima) to estimate the degree of positive staining for each individual marker as described previously⁵.

**Statistical analysis**

Data are presented as the means±SD from at least three independent experiments. The statistical significance of differences between two groups was
analyzed with Student's t-test. ANOVA and subsequent Bonferroni post-hoc analysis were applied to determined significance within multiple groups. Values of p < 0.05 were considered statistically significant.

References
Supplement to

Endothelial Nitric Oxide Synthase-Derived Nitric Oxide Prevents Dihydrofolate Reductase Degradation by Promoting S-Nitrosylation

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Supplemental Figures

Supplemental Figure I

Supplemental Figure I. eNOS inhibitor L-NAME dose-dependently reduces DHFR expression in HUVECs. L-NAME reduced DHFR expression from 1mM to 2mM range, but had no effect on GTPCH expression.
Supplemental Figure II. ONOO- does not alter DHFR expression in HUVECs. Tyrosine nitration of proteins as determined by 3-nitrotyrosine (3-NT) increased as the dose of ONOO- supplementation increased. ONOO- treatment reduced GTPCH expression in a dose-dependent manner, but had no effect on DHFR expression.
Supplemental Figure III. NO depletion reduces DHFR activity and BH4 levels in HUVECs. MG132 (1μM, 6h) prevented eNOS silencing caused DHFR activity (A) and BH4 contents (B) reduction. GSNO (100μM) supplementation reversed DHFR activity (C) and BH4 contents (D) reduced by eNOS silencing. MG132 (1μM, 6h) and GSNO (100μM) also prevented PTIO (150μM) induced DHFR activity (E and G) and BH4 contents (F and H) reduction in HUVEC. (n=3; *p<0.05 vs. Scr siRNA in A-D, or p<0.05 vs. control in E-H; #p<0.05 vs. eNOS siRNA in A-D, or p<0.05 vs. PTIO in E-H)
Supplemental Figure IV. S-nitrosylation of DHFR does not affect its activity in vitro. (A) GSNO (100μM) incubation increased S-nitrosylation of WT DHFR but not C7S DHFR in vitro. (B) Recombinant WT and C7S DHFR exhibited no difference in activities. GSNO incubation did not alter their activities.