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 degradated form were purchased from Merck (Temecula, CA). Protein A-Sepharose CL-4B beads were form 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% CO_2 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 8^{th} 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 °C. 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 previously[1].

Determination of BH_4 and Total Biopterins

The levels of BH_4 and total biopterins were determined via differential oxidation followed by high-performance liquid chromatography quantification, as described previously[2, 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 previously[3].

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 report[4].

Determination of 26S proteasome activity

Cells were incubated with 1μM of Me4BodipyFL-Ahx$_3$Leu$_3$VS (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% CO_2 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 previously[5].

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

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