Acute Inhibition of GTP Cyclohydrolase 1 Uncouples Endothelial Nitric Oxide Synthase and Elevates Blood Pressure

Shuangxi Wang, MD, PhD; Jian Xu, PhD; Ping Song, PhD; Yong Wu, MD, PhD; Junhua Zhang, MD, Hyoung Chul Choi, MD, PhD; and Ming-Hui Zou, MD, PhD

Division of Endocrinology and Diabetes, Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA

Supplemental materials and data:

Materials and Methods

Reagents

Polyclonal or monoclonal antibodies against GTPCH1, eNOS, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM1), and β-actin were obtained from Santa Cruz Biotechnology, and 3-nitrotyrosine (3-NT) antibody was from Upstate Biotechnology. Secondary antibodies were purchased from Cell Signaling Technology. Mouse GTPCH1 siRNA and control siRNA were purchased from Ambion RNA Company. Dihydroethidium (DHE) and diaminofluorescein (DAF) were purchased from Calbiochem (USA). Sepiapterin and 9,11-dideoxy-11-,9-epoxymethano-prostaglandin F2 (U46619) were obtained from Cayman Chemical. N-acetylserotonin (NAS), 2,4-Diamino-6-hydroxypyrimidine (DAHP), acetylcholine (Ach), sodium nitroprusside (SNP), and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Bovine aortic endothelial cells (BAECs) were obtained from Clonetics Inc. (Walkersville, MD).

Animals

Male wild-type (C57BL6) and $eNOS^{-/-}$ mice, 8-12 weeks of age and weighing 20-25 g, were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were housed in temperature-controlled cages under a 12 h light-dark cycle and given free access to water and normal chow. The siRNA sequences targeting murine GTPCH1 (GenBank™ accession number NM_008102) were as follows: 5'-GGGACAUUUUCUUCUUUAUtt-3' (sense) and 5'-AUAAAGAAGAAAAGUC CCtg-3' (antisense). Scrambled siRNA served as a negative control. Mice were transfected with siRNA as previously described.[1] Briefly, 25 μg of GTPCH1 siRNA was combined with in vivo-jetPEI™

(Polyplus Transfection, France) at N/P ratio of 5 (total volume, 100 μl). The mixture was incubated at room temperature for 15 min and retro-orbitally injected into mice. Seven days later, aortas were isolated to measure endothelium-dependent and -independent relaxation via organ chamber studies. Alternatively, aortas were used for biochemical assays described below. The animal protocol was reviewed and approved by the University of Oklahoma Institute Animal Care and Use Committee.

Cell culture

BAECs were grown in EBM (Clonetics Inc. Walkersville, MD) supplemented with 2% fetal bovine serum, penicillin (100 u/ml), and streptomycin (100 μg/ml). All cells were grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Experiments were performed on early-passage cells (passage 3 – 8) grown to at 70-80% confluence.

For isolation of primary mouse aortic endothelial cells (MAECs), collected aortas were washed twice with PBS at 4°C, carefully freed from all fat and connective tissue, and cut into 3 mm-long sections. These sections were then incubated in a 0.2% collagenase solution at 37°C with frequently shaking to detach endothelial cells from the aorta. MAECs were pelleted from solution through centrifugation at 1000 rpm for 15 min at 4°C, washed with PBS, and seeded onto culture plates containing EBM. Purity of MAEC cultures was confirmed through positive staining for eNOS, ICAM-1, and VCAM-1. MAECs at passages 3-5 were used for siRNA transfection.

Transfection of siRNA into endothelial cells

MAECs were transfected in 6-well plates according to a previously described protocol.[2] Briefly, a 10 μM stock solution of siRNA was prepared in 20 mM KCl, 6.0 mM HEPES (pH 7.5), and 0.2 mM $MgCl_2$. For each transfection, 100 μl transfection media (Gibcol) containing 4 μl siRNA stock solution was incubated with 100 μl transfection media containing 4 μl transfection reagent (Lipofectamine 2000, Invitrogen) for 30 min at room temperature. The siRNA-lipid complex was then added to each well, which contained 1 ml transfection media. After incubation for 6 h at 37°C, the transfection media was replaced with normal growth media, and cells were cultured for an additional 48 h.

Blood pressure measurement

Blood pressure was determined by a carotid catheter method.[3] Mice were anesthetized with a ketamine and xylazine mixture (70:6 mg/kg, intramuscular injection) and placed

under warm light (37°C). A catheter was inserted into the left common carotid artery, with the aid of a dissecting microscope, to measure arterial blood pressure. For catheter insertion, the left common carotid artery was carefully exposed via a 0.5- to 1.0-cm midline incision in the ventral neck region. The tip of the artery toward the head was ligated with a suture (5–0 silk), and the tip toward heart was occluded with a microclip (no. 18055–03; Fine Science Tool, Foster City, CA). A small cut was then made in the vessel wall using microscissors (no. 15000–08, Fine Science Tool). A 60-cm catheter (PE10 tubing, A-M Systems) containing a sterile 10% heparin-90% saline solution was inserted into the artery a distance of 0.65 cm toward the thorax. The arterial clip was removed, and the catheter was tied in place. Blood was directed to a pressure transducer through the catheter to obtain computerized blood pressure measurements (AD instruments). The mice were allowed to recover and the mean, systolic, and diastolic blood pressures were monitored for at least 30 min in conscious states.

Measurement of Biopterins

The levels of BH4 and total biopterins were determined as previously described with some modification.[4] Briefly, homogenates of aorta or cell lysates were suspended in distilled water containing 5 mM dithioerythrol, centrifuged at 12000g at 4°C for 10 min, and then subjected to oxidation in acid or base. To 100 μl aliquot of supernatant, 20 μl of 0.5 M HCl and 0.05 M iodine were added for acidic oxidation, and 20 μl of 0.5 M NaOH plus 0.05 M iodine were added for basic oxidation. After incubation for 1h in the dark at room temperature, 20 μl HCl was added to the basic oxidation only. All mixtures received 20 μl 0.1 M ascorbic acid for the reduction of excess iodine. Samples were then centrifuged for 10 min at 12000g at 4°C. Biopterin concentrations were determined by HPLC with a PR-C18 column. Elution was at a rate of 1.0 ml/min of 50 mM potassium phosphate buffer, pH 3.0. Fluorescence was detected with an excitation at 350 nm and emission at 440 nm. BH4 concentrations were calculated as the difference in results from oxidation in acid and base.

Detection of ROS

ROS production in culture cells or mice aortas was detected using the fluorescent probe DHE as described previously.[5] Briefly, before the end of treatment, 10 μM DHE was added to the medium and incubated for 30 min at 37°C, then washing with PBS twice.

The DHE fluorescent intensity in cells was recorded by fluorescent reader at the wave of excitation (485 nm) and emission (645 nm). The DHE fluorescence intensity in homogenates of aorta was assayed by HPLC according to the method we used before. Control was setup as 100%.

Detection of NO

NO production in culture cells was detected using the fluorescent probe DAF as described previously.[6] Briefly, before the end of treatment, 10 μM DAF was added to the medium and incubated for 30 min at 37°C, then washing with PBS twice. The DAF fluorescent intensity was recorded by fluorescent reader at the wave of excitation (485 nm) and emission (545 nm). Control was setup as 100%.

Organ chamber

Organ chamber study was performed as described previously.[7] Rings (3 mm in length) from mice aorta, free of fat and connective tissue, were mounted in organ bath by 2 stainless hook in 5 ml Kreb's solution (mM): NaCl 118.3, KCl 4.7, $MgSO_4$1.2, $KH_2PO_4$1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, EDTA 0.026 and glucose 11.0 at 37 ℃, gassed with 95%O_2+5%CO_2, under a tension of 0.8 g, for 1 h equilibration period. During this period, the Kreb's solution was changed every 15 min. After the equilibration, tissues were contracted with 60 mM high-potassium salt solution. After washing and another 30 minutes equilibration period, contractile response was evoked by U46619 (30 nM) to elicit reproducible responses. At the plateau of contraction, accumulative Ach (0.01, 0.1, 1, 10, 100 μM) or SNP (0.0001, 0.001, 0.01, 0.1, 1 μM) was added into the organ bath to induce the endothelium dependent or independent relaxation.

Western blot

Aortic tissues or endothelial cells were homogenized on ice in cell-lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed using specific antibodies. Band intensity (area × density) was measured by densitometry (model GS-700, Imaging Densitometer; Bio-Rad). Background intensity was subtracted from all calculated areas.

Reverse Transcription–Polymerase Chain Reaction

Total cellular RNA was isolated from mice aorta using RNA extract kit from invitrogen. The procedures for semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) were preformed by using forward (5'-GCCATGCAGTACTTCACCAA-3') and reverse (5'-AGGCTTCTGTGATGGCC ACCG-3') primers corresponding to murine GTPCH1 mRNA. Reactions were run for 30 cycles at conditions as follows: denaturation for 30 seconds at 94°C, annealing for 30 seconds at 57°C, and extension for 30 seconds at 72°C. Constitutively expressed GADPH mRNA was amplified as control.

Immunohistochemistry

Isolated aortas were immediately fixed in 4% paraformaldehyde. Immunohistochemical staining for 3-NT, ICAM1, and VCAM1 was performed as described previously.[8]

References

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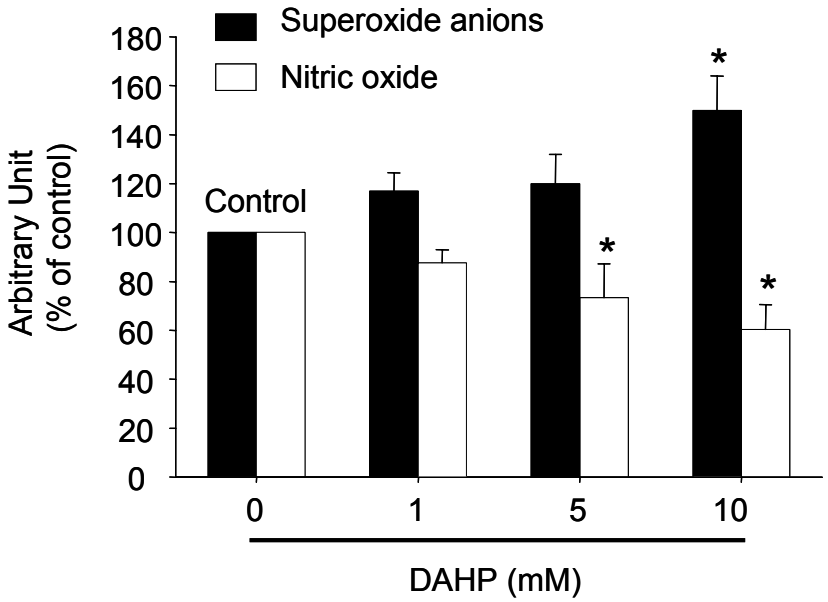

Figure S1. Inhibition of GTPCH1 by DAHP decreases NO release and increases $O_2^{\cdot-}$ productions in BAECs. BAECs were incubated with DAHP for 24 hours as indicated concentration. The NO release and $O_2^{\cdot-}$ productions were detected by DAF and DHE fluorescence respectively. Data are expressed as mean±SEM., N=5, **P*<0.05 DAHP vs. control.

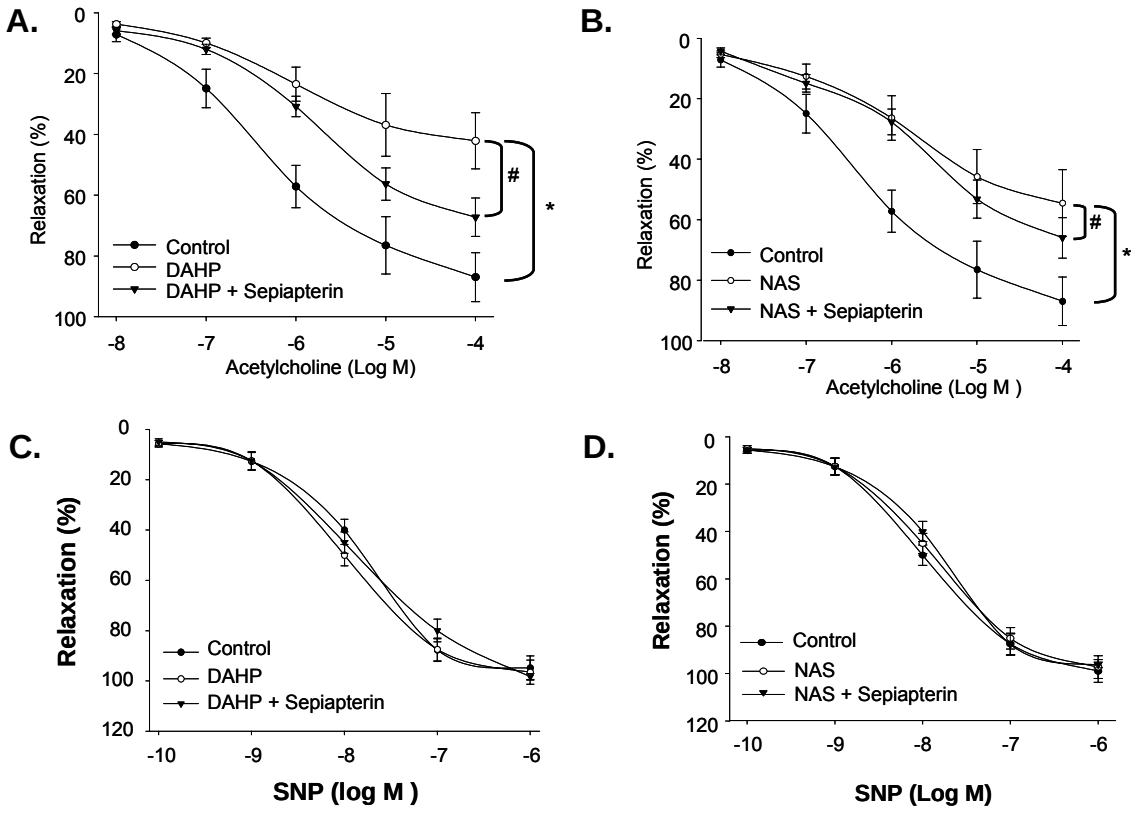

Figure S2. Pharmacological inhibition of GTPCH1 impairs the sepiapterin-reversible, endothelium-dependent relaxation in murine aortas. (A) Ach-induced endothelium–dependent relaxation of aortic rings incubated with EBM with 2% FBS for 24 h in the presence of DAHP (10 mM) ± sepiapterin (10 μM). Data are expressed as mean±SEM. N=6, **P*<0.05 DHAP vs. control; $^{\#}P$<0.05 DHAP vs. DHAP plus sepiapterin. (B) Endothelium–dependent relaxation of aortic rings incubated with EBM with 2% FBS for 24 h in the presence of NAS (1 mM) ± sepiapterin (10 μM). Data are expressed as mean±SEM. N=6, **P*<0.05 NAS vs. control; n=6 $^{\#}P$<0.05 NAS vs. NAS plus sepiapterin. (C) and (D) SNP-induced endothelium–independent relaxation of aortic rings incubated with EBM with 2% FBS for 24 h in the presence DAHP or NAS ± sepiapterin. Data are expressed as mean±SEM.

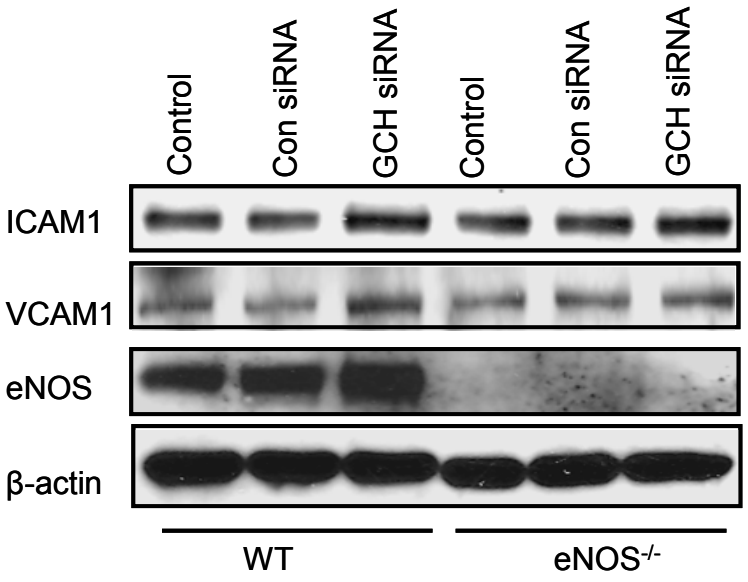

Figure S3. ***In vivo*** **GTPCH1 knockdown induces eNOS-dependent increases of ICAM-1 and VCAM-1.** Aortas from control or GTPCH1 siRNA-injected wild type (WT) or $eNOS^{-/-}$ mice were homogenated. The homogenates were subjected to perform western blot analysis of VCAM-1 and ICAM-1. The blot is a representative of at least three blots from three independent experiments.