SUPPLEMENTAL MATERIAL for

A Cell-Autonomous Role for Endothelial GTP Cyclohydrolase 1 and Tetrahydrobiopterin in Blood Pressure Regulation

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SUPPLEMENTAL METHODS

Genotyping *GCH1*$^{fl/fl}$; Tie2cre loci

Mice were genotyped by polymerase chain reactions using DNA prepared from ear biopsies. For *GCH1*$^{fl/fl}$ genotyping, PCR was performed using the following primers: *GCH1*$^{fl/fl}$-Fw 5'-GTC CTT GGT CTC AGT AAA CTT GCC AGG-3', *GCH1*$^{fl/fl}$-Rv 5'-GCC CAG CCA AGG ATA GAT GCA G-3'. The *GCH1* floxed allele showed a 1030 bp. For Tie2cre genotyping, PCR was performed using the following primers: Tie2cre Fw 5'-GCA TAA CCA GTG AAA CAG CAT TGC TG-3'. Tie2cre Rv 5'-GGA CAT GTT CAG GGA TCG CCA GGC G-3'. The Tie2cre allele amplified as 280 bp fragment.

Isolation of Murine Endothelial Cells

Primary lung/heart endothelial cells were isolated using MACS beads (Miltenyi Biotec), as described previously[1]. Briefly, mice were culled by overdose of inhaled isoflurane. Lungs/heart were harvested and digested in DMEM containing 0.18 unit/ml Liberase TM (Roche) and 0.1 mg/ml Dnase I (Roche) for 1 hour at 37°C. The digested tissue was filtered through 100 μm and 70 μm cell strainers. The cell suspension was then incubated with rat anti-CD31 antibody (BD Pharmingen) for 15 minutes at 4°C then with anti-rat secondary antibody coated immune magnetic beads for further 15 minutes at 4°C. Bead-bound endothelial cells were selected using a magnetic column. All of experiments, endothelial cells were cultured for 5 days in getatin coated 12 well plates in EBM endothelial cell media.

Genomic DNA production and excision PCR

Genomic DNA for detection of the excised allele was produced using the QIAamp kit (Qiagen). The floxed and excised allele were detected using the following primers: 5'GTC CTT GGT CTC AGT AAA CTT GCC AGG3', 5'GCC CAG CCA AGG ATA GAT GCA G3', and 5'GCT CAT CCC CCA CAC TTG TCT T3'. The *GCH1* floxed allele yields a1030 bp and the excised allele a production of 1392 bp.

Determination of Tissue Tetrahydrobiopterin Levels

BH4 and oxidised biopterins (BH2 and biopterin) were determined by high-performance liquid chromatography (HPLC) followed by electrochemical and fluorescent detection, respectively, following established protocol[2]. Briefly, snap frozen tissues (heart, lung, kidney, and liver) were homogenised and aortas were freeze-thawed in ice-cold resuspension buffer (50 mM phosphate-buffered saline, 1 mM dithioerythriol, 1 mM EDTA, pH 7.4). After centrifugation at 13,200 rpm for 10 min at 4°C, supernatant was removed and ice-cold acid precipitation buffer (1 M phosphoric acid, 2 M trichloroacetic acid, 1 mM dithioerythritol) was added. Following centrifugation at 13,200 rpm for 10 min at 4°C, the supernatant was removed and injected onto the HPLC system. Quantification of BH4 and oxidised biopterins was obtained by comparison with external standards and normalised to protein concentration, determined by the BCA protein assay.

Quantification of Intracellular Superoxide Production by Dihydroethidine HPLC

Superoxide production was quantified by measuring production 2-hydroxyethidium from dihydroethidium using HPLC[3, 4]. Briefly, primary endothelial cells were pre-incubated with serum-free DMEM (PAA) with or without 100 μM L-NAME (Sigma). Cells were then incubated with 25μM DHE (Invitrogen) for 20 min before being harvested for separation of DHE using gradient HPLC system (Jasco, UK) with an ODS3 reverse phase column (250mm, 4.5mm, Hichrom UK) and quantified using a fluorescence detector set at 510 nm (excitation) and 595 nm (emission).

Oxidative Fluorescent Microtopography

Endothelial superoxide production in mouse aortic frozen sections (n=6 per group) was detected using dihydroethidium (DHE) and fluorescent microscopy, as previously described[5]. Briefly, thoracic aortas (n = 6 per group) were harvested and frozen in optimal cutting temperature compound (OCT) (VWR International Ltd). Cryosections (30 μm) were cut at -25°C using a cryostat and placed on poly-lysine coated glass slide. Sections were incubated in KHB with or without 1 mM L-NAME for 30 min at 37 °C, then with 2 μM dihydroethidium (Invitrogen) for 5 min at 37°C in darkness. Sections were washed with ice-cold KHB, cover-slipped and placed in darkness. Images (x60) were acquired using a laser confocal microscope, set at identical acquisition settings (488 nm Excitation and 585 nm Emission). Endothelial superoxide production was quantified using Image-Pro Plus software by measuring the sum of the red intensities of the cells on the luminal side of the internal elastic lamina divided by the length of the vessel. For each ring, mean fluorescence was calculated from each quadrant of the vessels to produce n=1.

Analysis of NO Synthesis by eNOS

NO synthesis by eNOS was assessed by conversion of ^{14}C L-arginine to citrulline, in the presence and absence of N-monomethyl-L-arginine (1 mM, Sigma), as described previously[6]. Briefly, primary endothelial cells were incubated for 4 hours at 37°C in 200 μl Krebs-HEPES buffer containing ^{14}C L-arginine (2 μl of 50 μCi/mL). Samples were run on a SCX 300 cation-exchange HPLC column (Sigma) with online scintillation detection. Background signals were corrected from samples with ^{14}C L-arginine alone without cells.

Nitrite and Nitrate Determination

Freshly harvested aortas were preincubated in the presence or absence of L-monomethylarginine (100 μM, Sigma) in Krebs-Henseleit buffer (120 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 2.5 mM CaCl2, 25 mM NaHCO3, and 5.5 mM glucose) at 37°C, then exposed to acetylcholine (1 μM) for 30 min. Nitrite and nitrate accumulation was measured in samples of buffer using the CLD88 NO analyzer (Ecophysics) and normalized to aortic dry weight.

Determination of Aortic NO Production by Electron Paramagnetic Resonance

Electron paramagnetic resonance (EPR) spectroscopy was used to quantify aortic NO production, as described previously[7]. Briefly, freshly harvested aortas were stimulated with acetylcholine (1 μM) in 100 μl Krebs-HEPES buffers, then incubated with colloid iron (II) diethyldithiocarbamate

[$Fe(DETC)_2$] (285 μmol/L) at 37°C for 90 minutes. After incubation, aortas were snap-frozen in a column of Krebs-HEPES buffer in liquid nitrogen, and EPR spectra were obtained using an X-band EPR spectrometer (Miniscope MS 200; Magnettech). Instrument settings were: centre-field (B_0) 3276G, sweep 115G, microwave power 10 mW, modulation frequency 100 kHz, amplitude modulation 8000 mG, sweep time 60 s, and number of scans 4. Signals were quantified by measuring the total amplitude, after correction of baseline, and after subtracting background signals from incubation with colloid $Fe(DETC)_2$ alone. Values were normalized to aortic dry weight.

Determination of Aortic H_2O_2 Production

Aortic H_2O_2 production was determined by Amplex red assay (Molecular Probes), as described previously[8]. Briefly, freshly harvested aortic rings (6 x 2 mm) were incubated in 50 μl KHB containing 2 μM Ach, at 37°C for 60 minutes in dark, with or without 100 μM L-NAME. After incubation, 50 μl of Amplex Red reagent mixture containing 20 mM Amplex red, 0.4 U/ml horseradish peroxidase. Buffer fluorescence was determined at excitation 530 nm and emission 590 nm. Background fluorescence (without vessels) was subtracted and normalized to aortic dry weight.

Measurement of Arterial Blood Pressure

Blood pressure in conscious wild-type and *GCH1*$^{fl/fl}$Tie2cre mice was measured using the Visitech^{R} tail-cuff plethysmography system following 5 days training and 3 days baseline periods. In unconscious mice, blood pressure was measured in anesthetized wild-type and *GCH1*$^{fl/fl}$Tie2cre mice using the Millar catheter system, as described previously[1].

Isometric Tension Vasomotor Studies

Vasomotor function was analyzed using isometric tension studies in a wire myograph (Multi-Myogrph 610M, Danish Myo Technology, Denmark). Briefly, vascular rings were isolated from the thoracic aorta. The aortic rings or 2nd mesenteric arteries (2 mm) were mounted on a wire myograph containing 5 ml of Krebs-Henseleit buffer (KHB [in mmol/l]: NaCl 120, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 5.5) at 37°C, gassed with 95% O_2/5% CO_2. After allowing vessels to equilibrate for 30 minutes, the optimal tension was set. All experiments with aortas were performed in the presence of 10 μM non-selective cyclooxygenase (COX) inhibitor indomethacin (to block prostacyclin synthesis). The vessel viability was tested using 60 mM KCl. Concentration-response contraction curves were established using cumulative half-log concentrations of phenylephrine or U46619. Vessels were washed three times with fresh KHB, equilibrated for 20 minutes, and then precontracted to approximately 80-90% of maximal tension with PE or U46619. Acetylcholine and SLIGRL were used to stimulate endothelium-dependent vasodilatations in increasing cumulative concentrations. Responses were expressed as a percentage of the precontracted tension. Finally, the NO donor sodium nitroprusside (SNP) was used to test endothelium-independent smooth muscle relaxation in the presence of L-NAME. All pharmacological drugs were pre-incubated at least 20 min before the dose-response curves were determined. PEG-catalase was used at 400 units/ml, apamin at 50 nM, charybdotoxin at 100 nM, 1H-(1,2,4)oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) at 500 nM, indomethacin at 10 μM, L-NAME at 100 μM and sepiapterin at 10 μM. All drugs used were purchased from Sigma Chemical Company except ODQ (Cayman Europe) and SLIGRL (Abcam).

Western Blot Analysis

Western blot analysis was performed to measure protein levels of GTPCH, Phospho-eNOS (Ser1177), Phospho-eNOS (Thr495), eNOS, catalase, MnSOD, EcSOD, Cu/ZnSOD, CD102 and Phospho-VASP (Ser239) and VASP in aortas and primary cells from wild-type and *GCH1*$^{fl/fl}$Tie2cre mice. Isolated thoracic aortas and 2nd and 3rd branches of mesenteric arteries from *GCH1*$^{fl/fl}$Tie2cre mice and wild-type littermates were harvested and snap frozen on dry ice and stored at -80°C. Extraction of aortic and mesenteric protein was performed in Cellytic buffer (Sigma) containing protease inhibitor (Roche) and phosphatase inhibitor cocktail (Roche). The extract mix of aortic protein underwent three cycles of freeze-thaw. For mesenteric arteries, tissue was homogenised using a glass homogeniser. The lysate was centrifuged at 13,200 rpm for 5 min at 4°C. Supernatant was then transferred to a new tube and stored at -20°C. The BCA protein assay (Pierce) was used to determine protein concentrations. Protein lysates were added with 4X sample buffer (Invitrogen) and 10X reducing agent (Invitrogen). Protein lysates (5-15 ug) were loaded into a NuPage 4-12% Bis-Tris gel, transferred to PVDF membrane, incubated with a 1:10,000 dilution of rabbit anti-mouse GTPCH antibody (Gifted by Prof Steve Gross). Other proteins were detected using a 1:5,000 dilution of mouse anti-mouse eNOS antibody (BD Transduction Laboratories), 1:500 dilution of rabbit anti-phospho Ser1177eNOS antibody (Cell Signalling), 1:500 dilution of rabbit anti-phospho Thr495eNOS antibody (Cell Signalling), 1:1,000 dilution of rabbit anti-phospho VASP antibody (Cell Signalling), 1:1,000 dilution of rabbit anti VASP antibody (Cell Signalling), 1:500 dilution of goat anti-mouse CD102 antibody (R&D Systems), 1:2,500 dilution of rabbit anti-catalase antibody (Calbiochem), 1:2,500 dilution of rabbit anti-MnSOD antibody (Stressgen Bioreaents), 1:2,500 dilution of rabbit anti-ecSOD antibody (Stressgen Bioreaents), 1:2,500 dilution of rabbit anti-Cu/ZnSOD antibody (Stressgen Bioreaents), 1:20,000 dilution of mouse anti-GAPDH antibody (Millipore), 1:20,000 dilution of rabbit anti-beta tubulin antibody (Abcam) followed by appropriate HRP-conjugated secondary antibody (Promega). Protein bands were visualised by enhanced chemiluminescence (Super West Pico Chemiluminescence, Thermo Scientific).

Statistical analysis

Data are expressed as mean ± SEM. Comparisons between WT and *GCH1*$^{fl/fl}$Tie2cre were made by unpaired *Student* t test. Concentration-response curves were compared by ANOVA for repeated measurements. A p-value of less than 0.05 was considered statistically significant.

SUPPLEMENT REFERENCES

1. Bendall JK, Rinze R, Adlam D, Tatham AL, de Bono J, Wilson N, Volpi E, Channon KM. Endothelial nox2 overexpression potentiates vascular oxidative stress and hemodynamic response to angiotensin ii: Studies in endothelial-targeted nox2 transgenic mice. *Circ Res*. 2007;100:1016-1025.
2. Cai S, Alp NJ, McDonald D, Smith I, Kay J, Canevari L, Heales S, Channon KM. Gtp cyclohydrolase i gene transfer augments intracellular tetrahydrobiopterin in human

endothelial cells: Effects on nitric oxide synthase activity, protein levels and dimerisation. *Cardiovasc Res*. 2002;55:838-849.
3. Crabtree MJ, Channon KM. Dihydrofolate reductase and biopterin recycling in cardiovascular disease. *J Mol Cell Cardiol*. 2009;47:749-751.
4. Crabtree MJ, Tatham AL, Hale AB, Alp NJ, Channon KM. Critical role for tetrahydrobiopterin recycling by dihydrofolate reductase in regulation of endothelial nitric-oxide synthase coupling: Relative importance of the de novo biopterin synthesis versus salvage pathways. *J Biol Chem*. 2009;284:28128-28136.
5. Bendall JK, Alp NJ, Warrick N, Cai S, Adlam D, Rockett K, Yokoyama M, Kawashima S, Channon KM. Stoichiometric relationships between endothelial tetrahydrobiopterin, endothelial no synthase (enos) activity, and enos coupling in vivo: Insights from transgenic mice with endothelial-targeted gtp cyclohydrolase 1 and enos overexpression. *Circ Res*. 2005;97:864-871.
6. de Bono JP, Warrick N, Bendall JK, Channon KM, Alp NJ. Radiochemical hplc detection of arginine metabolism: Measurement of nitric oxide synthesis and arginase activity in vascular tissue. *Nitric Oxide*. 2007;16:1-9.
7. Khoo JP, Alp NJ, Bendall JK, Kawashima S, Yokoyama M, Zhang YH, Casadei B, Channon KM. Epr quantification of vascular nitric oxide production in genetically modified mouse models. *Nitric Oxide*. 2004;10:156-161.
8. Dikalov S, Griendling KK, Harrison DG. Measurement of reactive oxygen species in cardiovascular studies. *Hypertension*. 2007;49:717-727.

SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

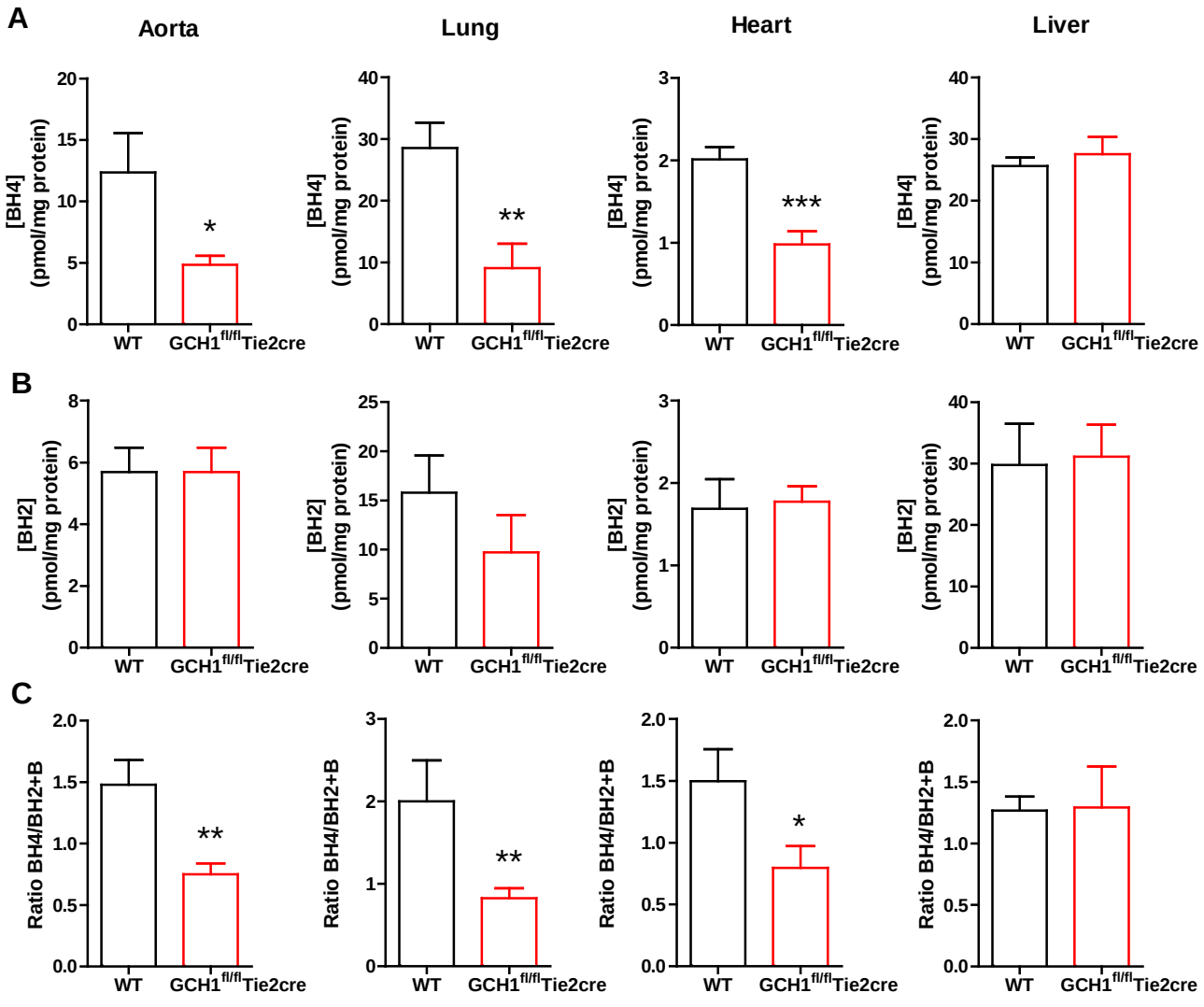

Figure S1. Quantitative analysis of tetrahydrobiopterin (BH4) metabolism in aorta, lung, heart and liver of wild-type (WT) and *GCH1*$^{fl/fl}$Tie2cre mice. Bar graphs showing levels of **(A)** BH4, **(B)** 7,8-dihydrobiopterin (BH2), and **(C)** BH4/BH2 + biopterin (B) ratio in aorta, lung, heart and liver of WT and *GCH1*$^{fl/fl}$Tie2cre mice. (**P*<0.05; ***P*<0.01; ****P*<0.001 n=6 to 10 animals per group)

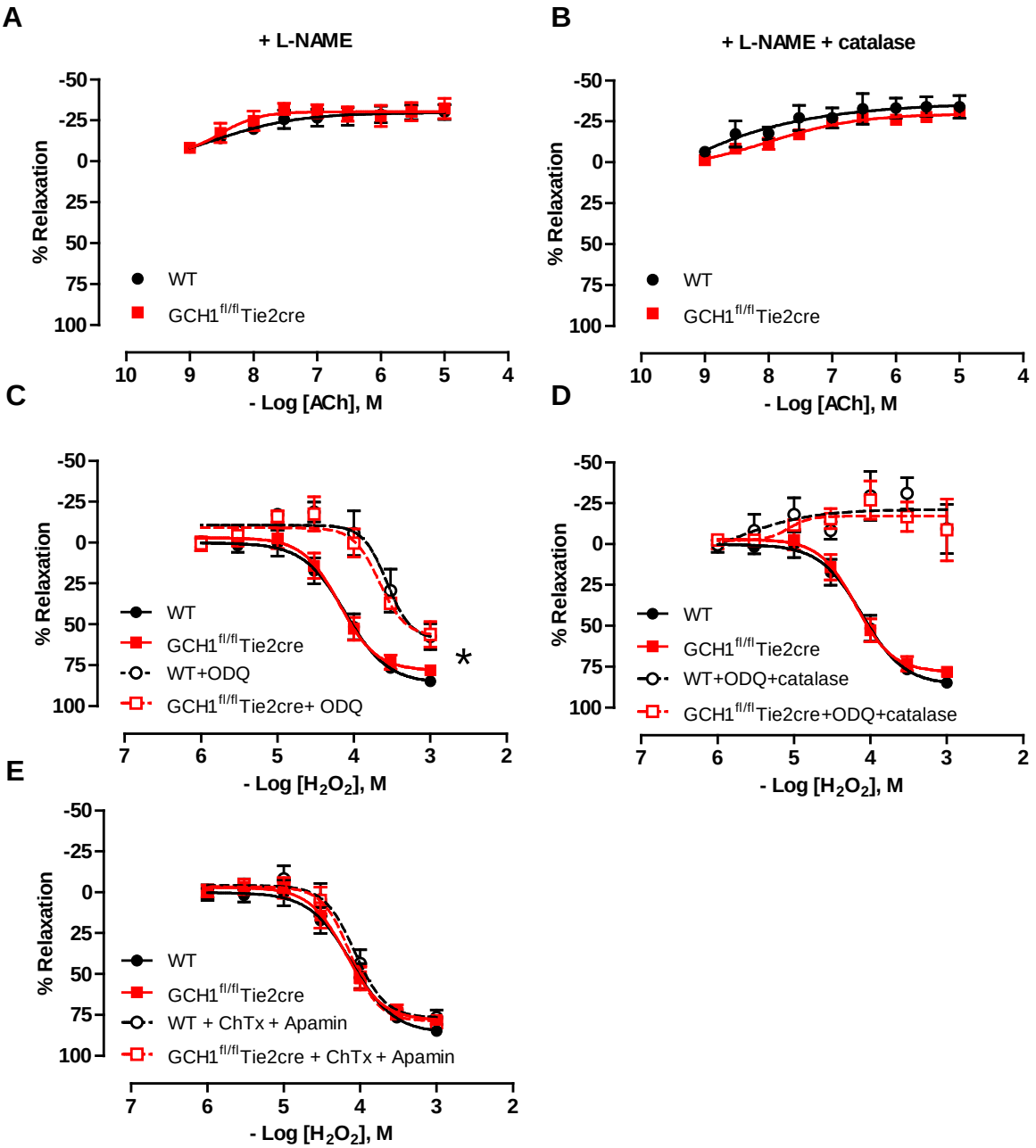

Figure S2. Endothelium-dependent vasodilatation to acetylcholine in aortas from wild-type (WT) and *GCH1*$^{fl/fl}$Tie2cre aortas in the presence of **(A)** L-NAME (100 μM), **(B)** L-NAME and PEG-catalase (400 unit/ml) (n=6 to 9 animals per group). **(C)** H_2O_2-induced vasodilatation in WT and *GCH1*$^{fl/fl}$Tie2cre aortas in the presence or absence of ODQ, **(D)** ODQ and PEG-catalase, and (E) charybdotoxin (ChTx) and apamin. (*P<0.05; n=4 to 6 per group).

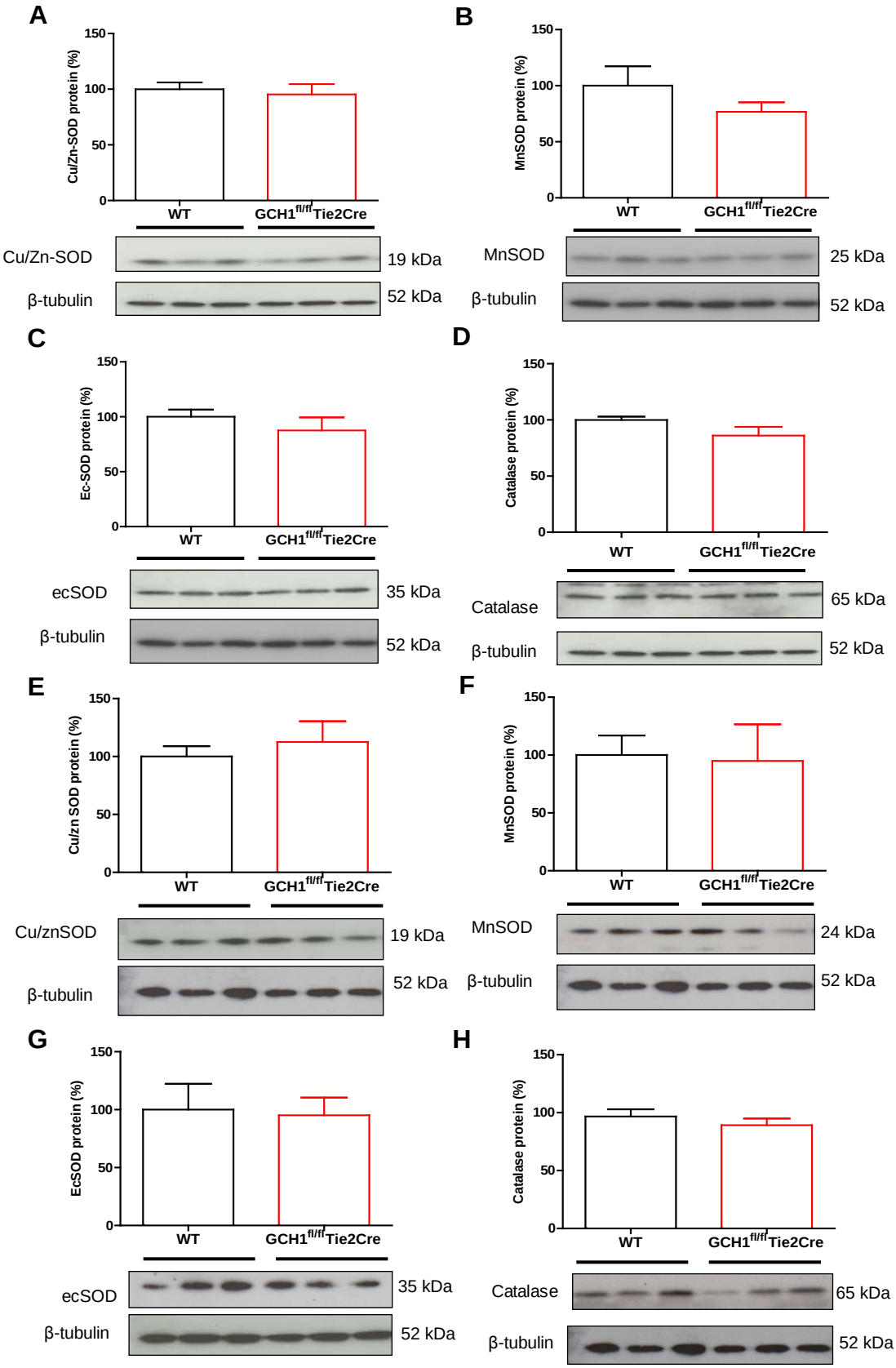

Figure S3. Representative immunoblots showing **(A)** Cu/ZnSoD, **(B)** MnSOD , **(C)** ecSOD, and **(D)** catalase protein in wild-type (WT) and *GCH1*$^{fl/fl}$Tie2cre aortas (n=6 per group) and mesenteric arteries in **(E)**, **(F)**, **(G)**, and **(H)** respectively, with quantitative data, measured as percentage band density, above, and corresponding beta-tubulin immunoblots, below (n=6 per group).

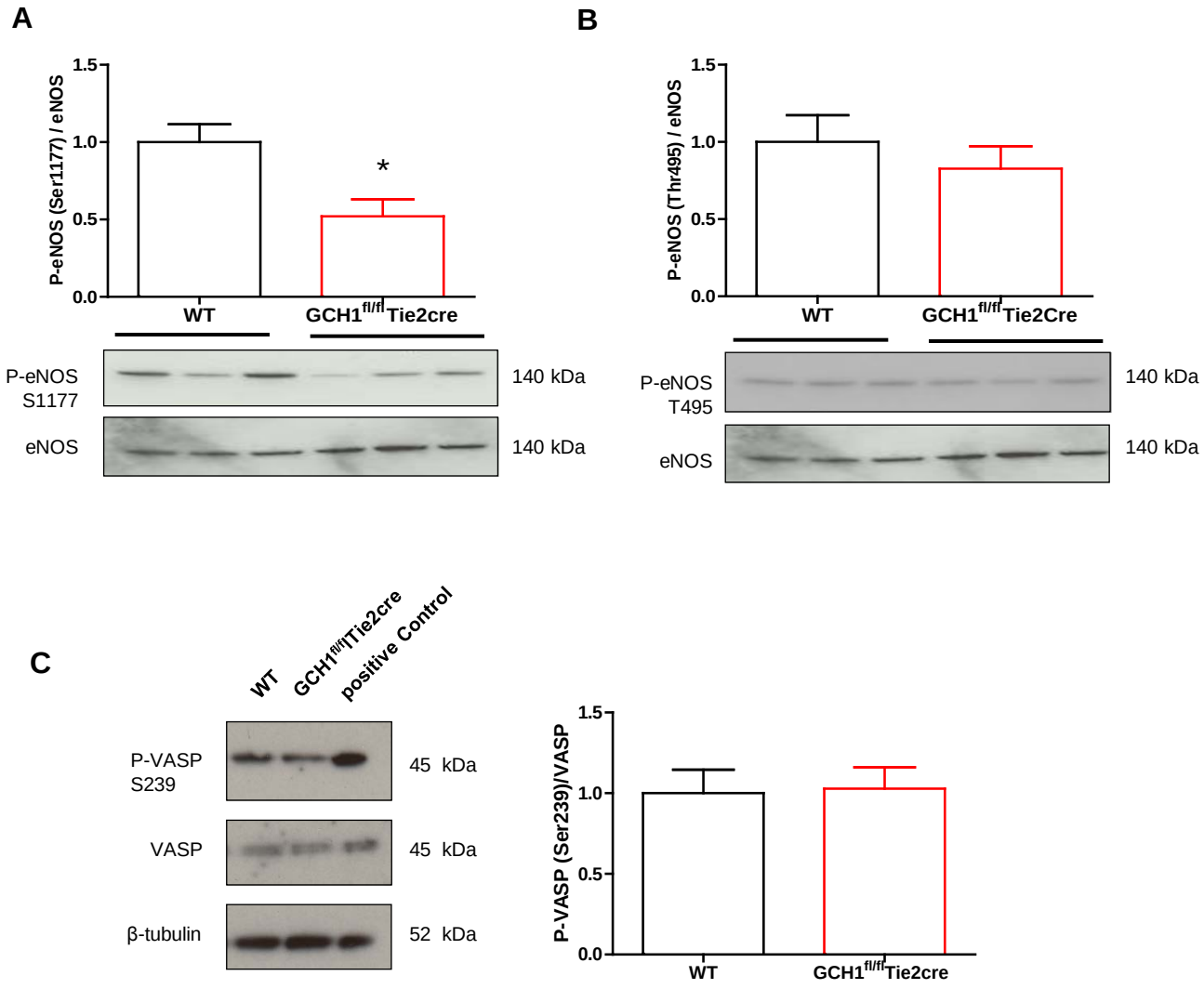

Figure S4. Representative immunoblots showing **(A)** Phosphorylation of eNOS at Ser1177 (P-eNOS S1177) to total eNOS protein in wild-type (WT) and *GCH1*$^{fl/fl}$Tie2cre aortas, **(B)** Phosphorylation of eNOS at Thr495 (P-eNOS T495) to total eNOS protein (**P*<0.05; n=6 per group). **(C)** Phosphorylation of vasodilator-stimulated phosphoprotein at serine 239 (P-VASP S239) and total VASP in freshly isolated aortas pre-incubated with acetylcholine (Ach, 0.5 µM) for 15 min at 37°C from WT and *GCH1*$^{fl/fl}$Tie2cre mice (left panel). Quantitative data (right panel), measured as band density of P-VASP to total VASP (n=6 per group). β-tubulin was used as loading control. Aorta stimulated with 1 µM sodium nitroprusside was used as positive control for pSer239-VASP.

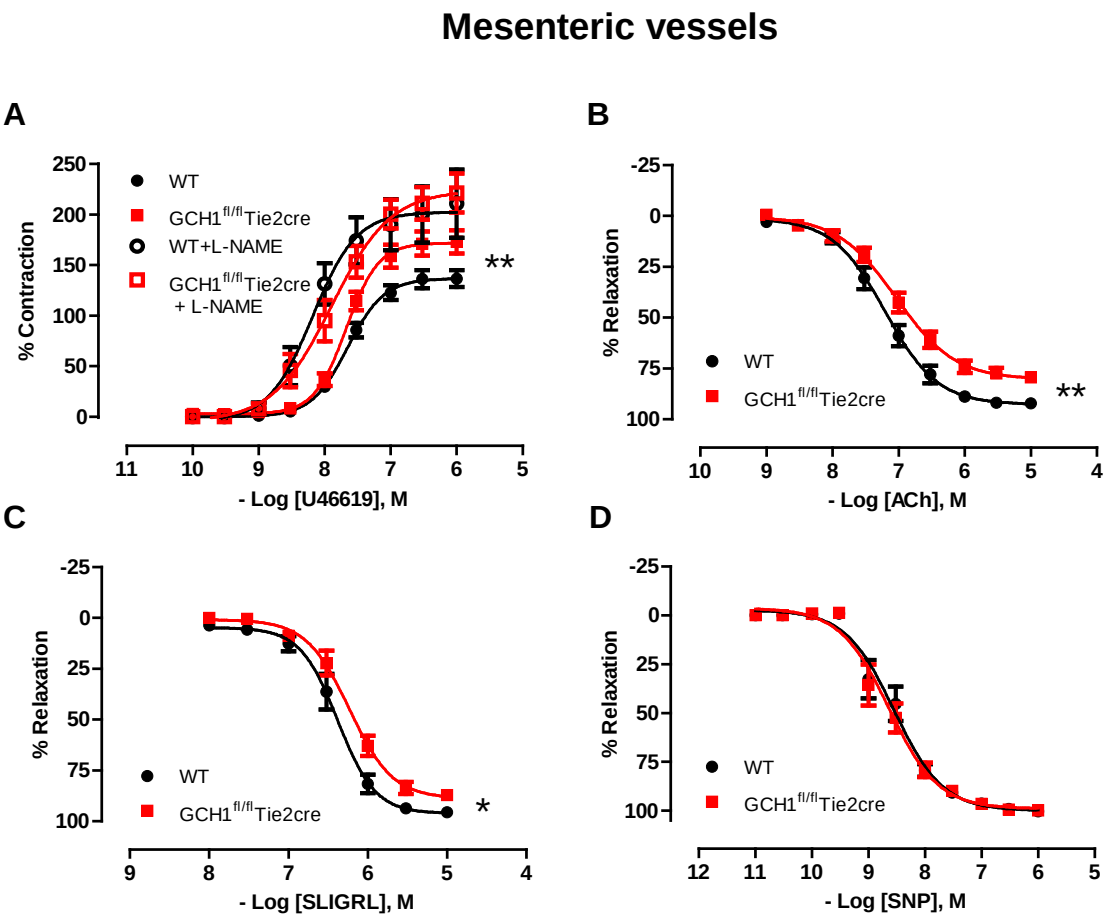

Figure S5. Vasomotor function in isolated 2nd order mesenteric arteries from *GCH1*$^{fl/fl}$Tie2cre and wild-type (WT) littermates. **(A)** Vasoconstriction to U46619 in the presence and absence of L-NAME. **(B)** Endothelium-dependent vasodilatation to acetylcholine and **(C)** SLIGRL. **(E)** Endothelium-independent vasodilatation to SNP. (**P*<0.05, ***P*<0.01; n=14 to18 animals per group).

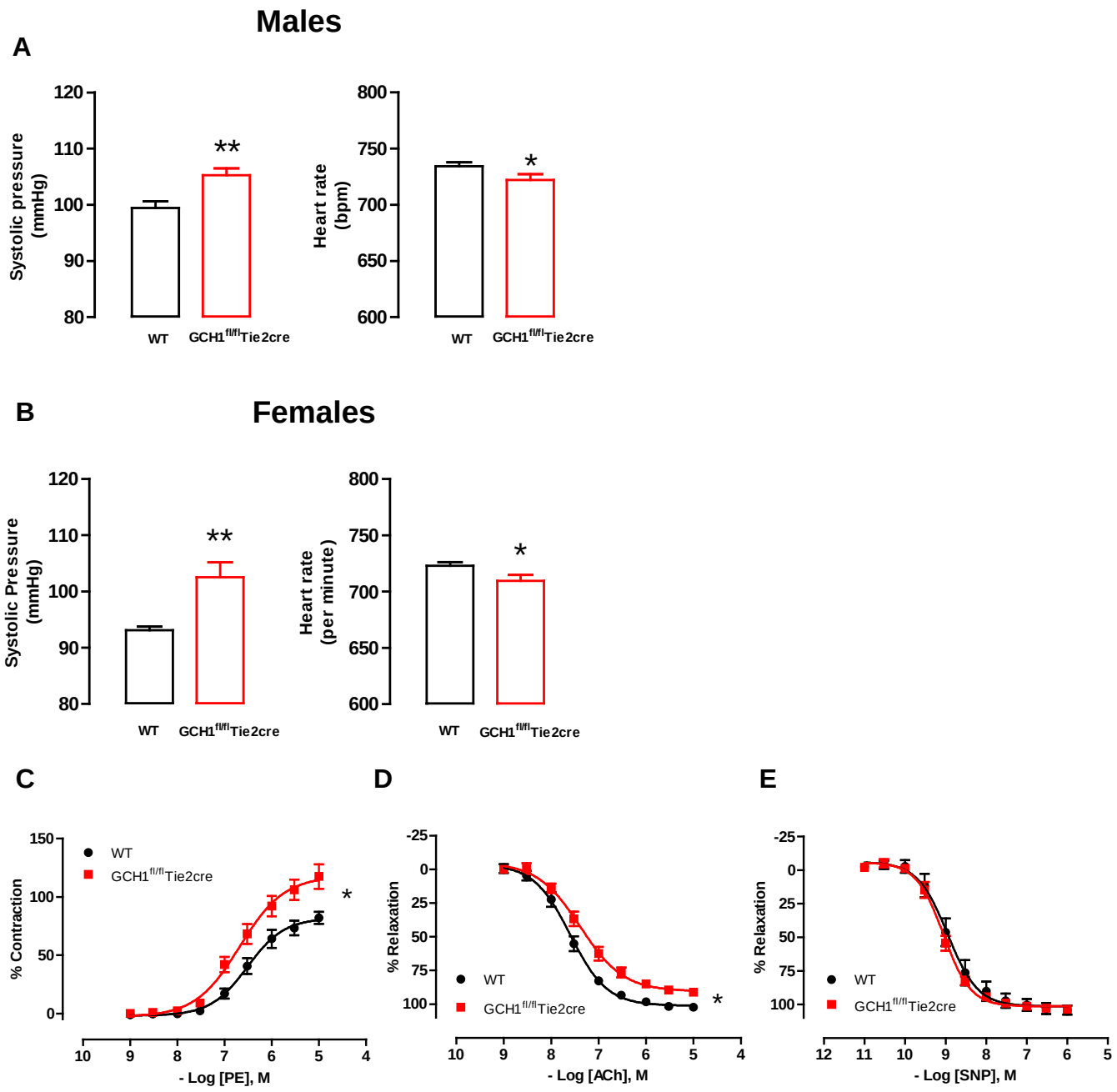

Figure S6. Systolic blood pressure and heart rate measured by tail-cuff method in **(A)** males **and (B) females .** Vasoconstriction to phenylehprine was enhanced in aortas from *GCH1*$^{fl/fl}$Tie2cre females compared to wild-type littermates (C). Endothelium-dependent vasodilatation to acetylcholine was significantly impaired in aortas from female *GCH1*$^{fl/fl}$Tie2cre mice compared to wild-type littermates. Endothelium-independent vasodilatation in response to sodium nitroprusside (SNP) was similar between the group (**P*<0.05; ***P*<0.001; n=8 to 10 animals per group)

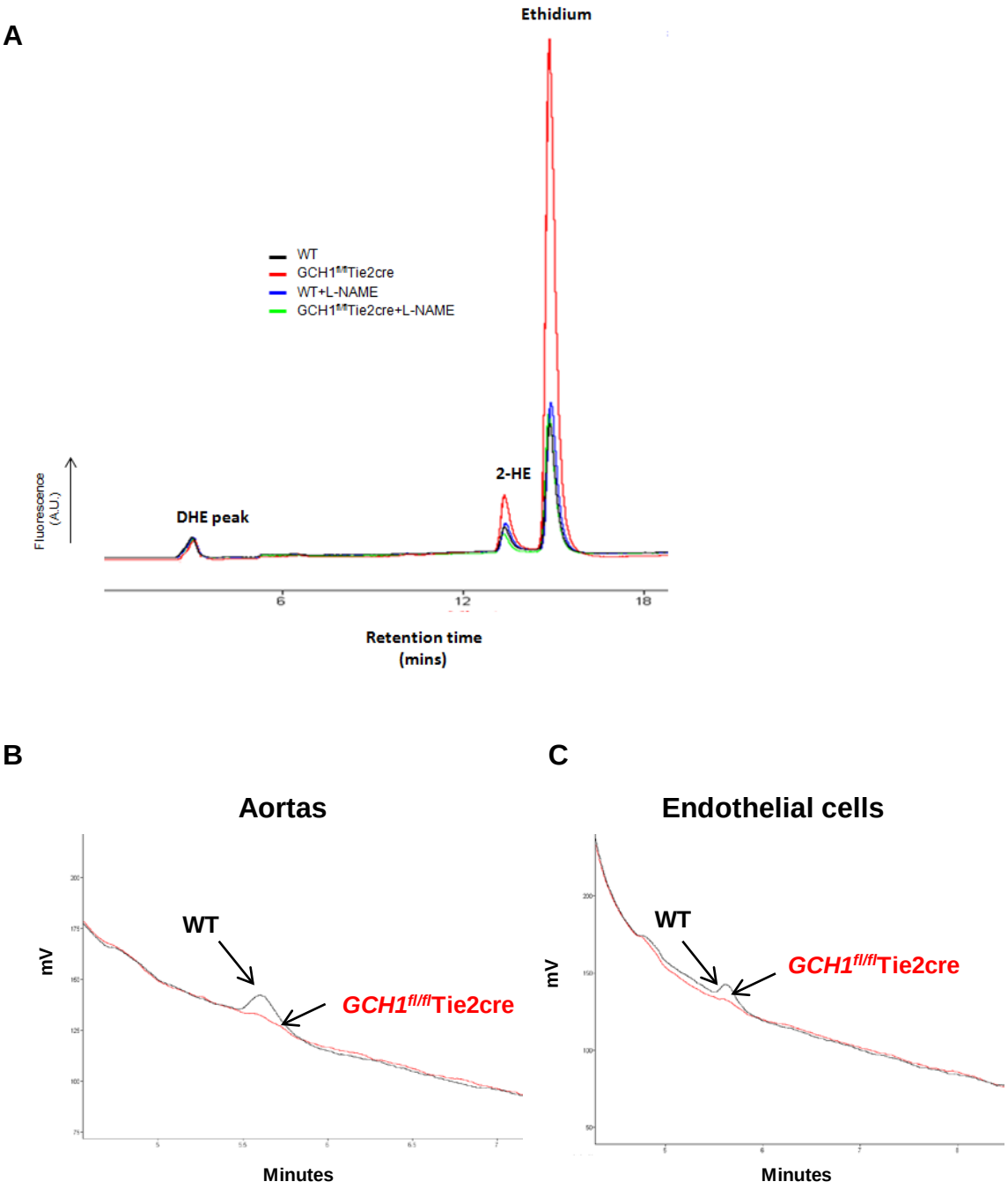

Figure S7. (A) Representative trances of 2-hydroxyethidium (2-HE) and ethidium peaks in primary endothelial cells isolated from wild-type (WT) and *GCH1*$^{fl/fl}$Tie2cre mice detected by DHE HPLC in the presence and absence of L-NAME. **(B)** Representative chromatograms of BH4 trances in aortas and **(C)** endothelial cells from WT and *GCH1*$^{fl/fl}$Tie2cre mice.