Materials and Methods

Materials

Phenylephrine (PE, P6126), acetylcholine (Ach, A6625), sodium nitroprusside (SNP, S0501), N (G)-nitro-L- arginine methyl ester (L-NAME, N5751) and L-Arginine (L-Arg, A8094) and NADPH (N0411) were purchased from Sigma. Purified rat anti-CD45 antibody (clone 30-F11) and Ter-119 (550565) were from BD Pharmingen (San Jose, CA). Anti-platelet serum (AIAD31440) was from Accurate Chemical (Westbury, NY). Bovine recombinant eNOS (60880) and the NOS activity assay (781001) were obtained from Cayman Chemical. Purified mouse anti-human eNOS antibody (610296) and rabbit anti-human eNOS was from BD Biosciences (San Joes, CA). Rabbit polyclonal anti-eNOS antibody (Ab66127) was from Abcam Inc. (Cambridge, MA). L-[^{14}C] arginine (NEC267E050UC) was from Perkin Elmer (Waltham, MA).

Blood collection and sample preparation

Blood was taken from the carotid artery and anticoagulated with citrate (for immunoprecipitation) or with heparin (for loading with fluorescent probes). All experiments were initiated within 2 hours of blood withdrawal. Whole blood was obtained via the carotid artery or inferior vena cava of donor mice (C57Bl/6J and Harvard eNOS$^{-/-}$).

The following manipulations were used to separate platelet rich plasma and hemoglobin from whole blood. In brief, blood was collected in a polypropylene tube containing 0.1 mL acid citrate dextrose buffer (Sigma) and then centrifuged at 120g, 4°C for 8 min and then at 14000 rpm, 4°C for 2 min. Between centrifugations, platelet rich plasma and buffy coat were removed by pipette and discarded. Leukocyte and platelet contamination were quantified with the aid of a hemocytometer and light microscope and did not exceed 0.05%. Leukocytes (25 uL blood sample) were stained by addition of 465 µL 3% citric acid and 10 µL 1% crystal violet (Sigma). Platelets (20 uL blood sample) were stained with the Unopette System (Becton Dickinson).

For protein identification, whole blood was collected in a syringe and centrifuged at 800 g for 15 min at room temperature (RT) to sediment RBCs prior to elution from the bottom of the syringe. Purity of the RBC preparations was confirmed using flow cytometry (FACS CANTO II; BD Bioscience, San Jose, CA, USA) and antibodies (as per manufacturer guidelines) specific for CD235 (glycophorin) as a RBC marker, CD45 as a leukocyte marker, and CD42 as a platelet marker.

Cells

Human umbilical vein endothelial cells (Promocell GmbH; Heidelberg, Germany) were cultured in 10 cm-diameter Petri plates (passages 1-4) using a commercial endothelial cell basal medium (Promocell GmbH, Heidelberg, Germany) supplemented with penicillin and streptomycin (*PAA* Laboratories GmbH, Cölbe, Germany). Cell pellets were obtained after detachment with trypsin.

Animals

Wild type C57BL/6 and B6.SJL-PTPRCPEP/BOY, B6.129P2-Nos3tm1Unc/J (UNC eNOS$^{-/-}$) and B6.129P2-NOS2 TM1 LAU/J (iNOS$^{-/-}$) were from Jackson Laboratories (Bar Harbor, ME).

Breeder stocks of Harvard eNOS$^{-/-}$ mice, backcrossed 10 generations to C57BL/6 mice, were from Dr. Paul L. Huang (Harvard University).[1] Homozygous matings produced offspring for this study. All animals were housed under pathogen-free conditions and only male mice were used for experiments. eNOS immunoprecipitation experiments in mouse red blood cells, as well as aortic cGMP studies were conducted using Düsseldorf eNOS$^{-/-}$ mice from Dr. Axel Gödecke (Heinrich Heine University of Düsseldorf).[2] Genetic identity of animals was routinely confirmed by PCR analysis of tail clip DNA using gene-specific probes. All surgical procedures were reviewed, approved and performed according to the criteria outlined in the NHLBI Animal Care and Use Committee and the LANUV Nordrhein-Westfalen guidelines. Mice were fed standard laboratory chow ad libitum until use in experiments.

Chimeras

Two congenic strains of WT mice on C57Bl/6 background (C57BL/6 and B6.SJL-PTPRCPEP/BOY) were used to mismatch BM donors and recipients for leukocyte antigen expression (CD45.2 versus CD45.1) to permit flow cytometric analysis of recipients' blood cell reconstitution to donor phenotype. BM cells were isolated from the femurs and tibias of donor mice and resuspended in sterile PBS to a 1-2 x10^7/mL final cell count. Recipients were lethally irradiated (two 500 rad doses, 3 hours apart). Following the second irradiation, 2-4 x10^6 donor BM cells in 200 µL of PBS were injected into the retroorbital sinus of each recipient. Chimeras were housed in autoclaved cages with 0.2% neomycin drinking water for 2 weeks, followed by normal drinking water. At 6-8 weeks post-transplant, chimeric mice were assessed by flow cytometry for conversion to donor phenotype prior to use in experiments.

Flow cytometry

Flow cytometry was used to verify reconstitution of BM transplanted chimeric mice. In brief, leukocytes (from 50 uL lysed whole blood) from chimeric mice were stained in vitro with FITC-labeled anti-CD45.1 (PharMingen, Inc.) and PerCP-Cy5.5 anti-CD45.2 antibodies (PharMingen, Inc.) and immediately analyzed by flow cytometry (BD FACSCaliber) to determine relative expression of CD45.1 versus CD45.2. Leukocytes were gated based on their size (forward light scatter) and granularity (side light scatter) in a double logarithmic scatter dot plot. The median fluorescence intensity (MFI) of 10,000 events within the leukocyte population was determined by analyzing the distribution histogram obtained by plotting green fluorescence intensity (FITC channel) against far-red fluorescence intensity (PerCP-Cy5.5 channel).

Immunoprecipitation, gel electrophoresis and western blot analysis

Mouse RBCs, mouse aorta and human endothelial cells were lysed with RIPA lysis buffer containing protease inhibitor cocktail (Roche Applied Science), as previously described.[3] Total protein concentration was determined by the Lowry assay (DC Protein Assay, Bio-Rad). For direct immunoprecipitation (IP), antibodies were purified from preservatives and contaminants by using Protein G coupled dynabeads (Invitrogen) and concentrated using an ultrafiltration column (Millipore) according to the manufacturer's' instructions. The antibodies were then cross-linked to Epoxy-Dynabeads (Invitrogen). RBC or aortic lysate in RIPA Buffer [4] was incubated overnight with crosslinked Dynabeads at 4°C, followed by washing and elution with loading buffer (Invitrogen). For gel electrophoresis, samples were loaded in Bis-Tris gel, 4-12%

(aorta from chimeras) or 3-8% or 7% NuPAGE Novex Tris/Acetate pre-cast gels (Invitrogen). For western blot analysis, proteins were transferred onto polyvinylidene fluoride (PVDF) membrane Hybond P (Amersham Biosciences, Munich, Germany), using a pre-stained protein ladder (PageRuler Plus, Fermentas Life Science) to control for the transfer. The membrane was blocked with 5% nonfat dry milk (Bio-Rad) in TBS (10 mM Tris, 100 mM NaCl), incubated with a mouse anti-human (overnight 4°C 1:500) or rabbit anti-eNOS antiserum (BD Bioscience) diluted (1 h RT 1:1000) in T-TBS (0.1% Tween in TBS), washed for 30 min in T-TBS, and then incubated with HRP-conjugated goat anti-mouse (1:5000 from Jackson Immuno Research Laboratories (chimeras) or BD Bioscience), or anti-rabbit antibody (1:5000 Rockland, PA, USA). The bands were visualized by autoradiography on Hyperfilm ECL (Amersham Biosciences) using SuperSignal West Pico or Femto Chemiluminescent Substrates (Pierce, Thermo Fisher Scientific,Waltham, MA, USA).

Real-Time reverse transcription polymerase chain reaction (RT-PCR)

Quantitative real-time RT-PCR was used to analyze gene expression of eNOS (NOS3), COX-1 and COX-2 in aortic tissue from C57Bl6/J, $eNOS^{-/-}$, and BM transplanted chimeric mice (groups: BC+/EC+, BC-/EC+, BC+/EC- and BC-/EC-). First-strand cDNA was synthesized on RNA (previously isolated from aortas) using random primers (Applied Biosystems; Norwalk, CT) in a reverse transcriptase reaction mixture (Superscript cDNA synthesis kit; Invitrogen, Carlsbad, CA). Quantitative real-time PCR assays were carried out with the use of gene-specific double fluorescently labeled probes (Applied Biosystems) in a 7900 Sequence Detector (PE Applied Biosystems) according to the manufacturer's instructions. In brief, PCR amplification was performed in a 384 well plate with a reaction mixture containing primer, probe, dNTP in real time PCR buffer and passive reference (ROX) fluorochrome, and the appropriate thermal cycling conditions. Samples were analyzed in triplicate and normalized to the housekeeping gene ß actin.

Immune-fluorescence staining

Freshly obtained whole blood was smeared across glass slides and allowed to air-dry for 20 min. Paraformaldehyde (4% in PBS) was then applied to the slides for 25 min. After washing, blocking and permeabilization were achieved by applying 5% Donkey serum in PBS with 0.05% Tween 20 for 1 hour. The blocking solution was then discarded and immune-fluorescence staining performed using primary antibodies against eNOS (#ab66127, Abcam) and Ter-119 (#550565, BD Pharmingen), both applied at 1:100 dilution for 1 hour. Control slides were routinely stained in parallel by substituting IgG, or the specific IgG isotype, from the same species for the primary antibody at the same final concentration. After brief washing, secondary antibodies (Rhodamine Red-X conjugated Donkey anti-Rat #712-296-153 and FITC conjugated Donkey anti-Rabbit #711-095-152; Jackson ImmunoResearch Laboratories) were applied for 1 hour, both at 1:200 dilution. After final washing, mounting media containing 4',6-diamidino-2-phenylindole (DAPI) (#H-1200, Vector Laboratories) and a cover slip were applied. Images were acquired using a Zeiss LSM 510 UV laser scanning confocal microscope system (Carl Zeiss GmbH). For WT, $eNOS^{-/-}$ and IgG controls, immune-fluorescence staining was performed at least eight times per group and using blood from ≥ 5 different mice.

Wire myography

Mice were anesthetized with ketamine chloride and xylazine (120 mg/kg and 6 mg/kg body weight i.p., respectively). After placing the mouse in the supine position, the right carotid artery was cannulated for systemic blood pressure measurement (PowerLab). The thoracic and abdominal cavities were opened through a midline incision. After achieving hemostasis, the thoracoabdominal aorta was carefully dissected, removed and placed in ice-cold physiologic salt saline (Krebs buffer). The composition of Krebs buffer was (mM): NaCl (119), KCl (4.5), $NaHCO_3$ (25), KH_2PO_4 (1.2), $MgSO_4$ (1.2), L-glucose (11) and $CaCl_2$ (2.5). Using a dissecting microscope, the attached fat and adventitia were meticulously removed by sharp dissection and clotted blood was flushed from the vessel lumen. Each aortic ring was used to generate a dose-response curve to test for: 1) contraction to phenylephrine (PE: 10^{-9} M to 10^{-4} M), 2) dilation to sodium nitroprusside (SNP: 10^{-9} M to $10^{-4.5}$ M), and 3) dilation to acetylcholine (Ach: 10^{-9} M to $10^{-3.5}$ M). For the determination of SNP and Ach dose-response relationships, aortic rings (3 mm length) were pre-contracted with 10^{-6} M PE. Endothelium-independent contraction was calculated as % contraction relative to maximal vessel tension (at the third 120 mMol KCl rinse). Endothelium-independent (SNP) and -dependent dilation (Ach) were calculated as % dilation relative to the pre-contraction tension (10^{-6} M PE). All experimental protocols were applied to C57Bl/6 mice and BM transplanted chimeras BC+/EC+ and BC-/EC+. Five mice (2 aortic rings per mouse) were included in each group.

Blood pressure measurements

Baseline blood pressures (mean arterial, systolic and diastolic) were measured under anesthesia (120 mg/kg ketamine and 6 mg/kg xylazine) via a heparinized catheter (in-line with a blood pressure transducer and using Powerlab software) surgically implanted in the right carotid artery of each mouse. Blood pressure was determined after a 20 min post-surgery stabilization period. Core body temperature was maintained at 35 ± 0.5°C.

The contribution of platelets and leukocytes to blood pressure was assessed in BC+/EC- and BC-/EC- chimeras treated with either anti-platelet serum (APS) or a purified rat anti-CD45 antibody (anti-CD45 Ab) to induce thrombocytopenia or leukopenia, respectively. Treatment with APS (62.5 µl/kg in 200 µl sterile PBS i.p. for 2 days) depleted circulating platelets by ≥ 90% and treatment with anti-CD45 antibody (dose: 1 mg/kg/day in sterile PBS i.p. for 2 days) depleted circulating leukocytes by ≥ 75%. Thrombocytopenia and leukopenia were confirmed by manual platelet and leukocyte counts, respectively. Neither treatment significantly altered hematocrits. Blood pressure measurements were made on the 2^{nd} or 5^{th} day following initiation of treatment with APS or anti-CD45 antibody, respectively.

Hemodynamic responses

BC+/EC- chimeras (made with Harvard $eNOS^{-/-}$ or UNC $eNOS^{-/-}$ mice) were surgically implanted (under temporary anesthesia: ketamine/xylazine or isoflurane) with a microminiaturized electronic monitor (PA-C10; Data Sciences International; St. Paul, MN, USA) attached to an indwelling aortic catheter. Digitized hemodynamic data were continuously sensed, processed and transmitted via radio frequency signals to a nearby receiver (acquisition period: 10 min every 2 hours). Mice were allowed a 10-day post-surgery stabilization period on

standard diet and drinking water before hemodynamic responses to NOS inhibition (L-NAME, 1g/L in drinking water for 4 days) or stimulation (L-Arginine, 2% in drinking water for 3 days) were assessed. Blood pressures were determined by averaging night-time data (22:00 to 4:00) on day 10 (baseline), day 14 (L-NAME) and day 17 (L-Arginine). Change in MAP is reported as mmHg ([L-NAME - baseline BP] and [L-Arg BP - L-NAME BP]).

cGMP levels in mouse aorta

The levels of cGMP in mouse aorta were assayed by using a DetectX High Sensitivity Direct cyclic GMP Immunoassay kit (Arbor Assay, Ann Arbor, MI, USA), following the manufacturer's instructions. The cGMP concentrations were normalized for protein content using the Lowry assay (DC Protein Assay, Bio-Rad).

Reductive chemiluminescence

Reductive chemiluminescence measurements were performed as previously described with minor modifications.[5, 6] Briefly, whole blood and plasma nitrite were measured in blood samples collected via carotid cannulation of anesthetized (120 mg/kg ketamine hydrochloride and 6 mg/kg xylazine) mice into sterile, nitrite-free, heparinized (0.5-1 IU) syringes. Whole blood nitrite was preserved by diluting it 1:4 in preservation solution (800 mM $K_3Fe(CN)_6$, , 10%, v/v Nonidet-40 substitute, and 100 mM *N*-ethylmaleimide. Plasma was obtained by centrifugation at 4°C and 14000 rpm for 2 min. Nitrite concentrations were determined by the tri-iodide assay[7] in a chemiluminescence NO• analyzer (Sievers NOA; Boulder, CO) using nitrite as a standard.[7]

NOS activity

RBC membrane extracts were prepared from platelet- and leukocyte-poor blood by 3 to 4 washing steps in 1 mL homogenization buffer (Cayman Chemical) and centrifugations at 14000 rpm, 4°C for 20 min to produce relatively hemoglobin-free RBC membrane preparations. NOS activity of 10 µl RBC membrane preparations was assayed by measuring the conversion of L-[^{14}C] arginine into L-[^{14}C] citrulline using a commercial kit (Cayman Chemicals), following the manufacturer's protocol with minor modifications. Reaction buffer (45 µL) contained 5 µL L-[^{14}C] arginine (concentration: 0.1mCi/mL, specific activity: >300mCi (11.1GBq/mmol), Perkin Elmer), 1 mM NADPH, 100 nM calmodulin, 2 mM $CaCl_2$ (final volume 65 µL). All samples were prepared in duplicate: 1) incubation at 37°C for 24 hours and 2) incubation at -20°C for 24 hours. After addition of 400 µL of stop buffer followed by freeze fracture at -20C for 20 min, L-[^{14}C] citrulline was eluted in 100 uL ion exchange resin and centrifuged at 2000 rpm, RT for 4 min. Proteins were precipitated using ice-cold methanol (600 µL) for 20 min at 20°C, and centrifugation for 2 min at 14000 rpm, to avoid color quenching. The increase in production of L-[^{14}C] Citrulline equivalents was calculated as follows:

$[WT_{37°C}$ cpm – $WT_{-20°C}$ cpm] / $[eNOS^{-/-}{}_{37°C}$ cpm – $eNOS^{-/-}{}_{-20°C}$ cpm].

The eNOS activity (fmol/min) was calculated as the conversion of the added radioactive L-[^{14}C] Arginine (cpm/fmol) into L-[^{14}C] Citrulline (cpm) during the considered reaction time.

Statistical analysis

All values are reported as mean ± SEM. Comparisons between groups were made using either Student's *t*-test or ANOVA followed by Bonferroni posthoc test for more than two group comparisons. Differences were deemed significant when $p < 0.05$. Statistical analyzes were performed using GraphPad Prism.

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