Methods

Animals

Wild type, TSP1 and CD47-null mice extensively backcrossed into a C57BL/6 background were housed under pathogen-free conditions and had ad libitum access to filtered water and standard rat chow. Experiments and handling and care of animals conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Reagents

Acetylcholine was purchased from Sigma-Aldrich (St. Louis, MO). TSP1 was isolated from fresh human platelets as previously described {Roberts, 1994 #204} or purchased from Athens Research & Technology (Athens, GA). The recombinant signature domain of TSP1 (E123CaG1) was kindly provided by Dr. Deane Mosher (University of Wisconsin at Madison). The murine CD47 monoclonal antibody clone 301 was provided by Dr. William A. Frazier (Washington University School of Medicine, St. Louis, MO). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). DETA/NO was kindly provided by Dr. Larry Keefer (NCI, Frederick).

Cell Cultures

Bovine aortic endothelial cells (BAEC) and human umbilical vein endothelial cells (HUVEC) were obtained from Lonza Group Ltd. (Switzerland) and maintained in manufacturer provided growth medium. Cells were used between passages 2 – 8.

Intracellular Cyclic Nucleotide Measurement

BAEC were starved overnight in endothelial basal medium plus 0.1% BSA prior to treatment. Intracellular cGMP levels were determined using an enzyme immunoassay (Amersham, GE Health Care, UK) as per the manufacture's instructions. Data were normalized to μg of protein with a BCA Assay kit (Pierce Biotechnology, Rockford, IL).

eNOS Activation Assay

BAEC were starved overnight in endothelial basal medium plus 0.1% BSA. On the day of treatment cells were incubated in assay incubation buffer (10 mmol/L HEPES, 145 mmol/L NaCl, 5 mmol/L KCl, 5 mmol/L $MgSO_4$, 10 mmol/L glucose, 1.5 mmol/L $CaCl_2$, 0.25% BSA, pH 7.4). Treatment agents were added for 15 min prior to cell stimulation using ACh (10 μM). Cells then received [^{3}H]-L-arginine (Perkins Elmer). The assay was terminated 3 min later by the addition of stop buffer (5 mmol/L L-arginine, 4 mmol/L EDTA, 4 mmol/L EGTA, 6 mmol/L L-citrulline in PBS). The cells were lysed with lysis buffer (5 mmol/L Tris-HCl (pH 7.4), 20 mmol/L EDTA, 0.5% Triton X-100), freeze-thawed twice, and the cell lysate was added to pre-equilibrated Dowex-50-H^+ columns. Equal volumes of eluent were mixed with 5 ml of scintillation cocktail, and ^{3}H-citrulline was quantified using a 1900CA Liquid Scintillation Analyzer (Packard). Lysate protein levels were determined using a BCA assay, and counts were normalized to protein levels. All conditions were assessed in triplicate, and all experiments were repeated at least three times. In other experiments cell lysates, prior to running through Dowex columns, were counted directly to measure L-arginine uptake.

Endothelial Cell Calcium Transients Measurement

Agonist-induced Ca^{2+} release was monitored in endothelial cells in the presence or absence of 2.2 nM TSP1 or other treatment agents using live cell imagining. For single-cell imaging, HUVEC were grown on gelatin-coated 35mm glass bottom dishes (MatTek Corp., MA) and serum starved with 0.01% BSA 24 hrs prior to experiments. Cells were loaded with 5umol/L Fluo-4AM ester (excitation at 488nm, Molecular Probes, Invitrogen) for 30 min at 37 C in HBSS (with 1.88 mmol/L $CaCl_2$ and 20 mmol/L HEPES) followed by 2 washes and incubation at room temperature in the dark for an additional 20 min. Images were acquired every 3 sec on a Zeiss LSM 5 confocal microscope (Carl Zeiss, Thornwood, NY) at 20X and a resolution of 256x256. Data were analyzed using software LSM Pascal 3.2 obtaining relative changes in fluorescence

intensity compared to baseline. We added plain buffer 1 min prior to addition of the agonist ionomycin (3 μmol/L). If cells showed changes in calcium flux due to buffer addition, they were excluded from the analysis. For en face calcium measurements in fresh aortas from wild type mice, vessel segments were loaded for 1 hr with 5 μM Fluo-4AM, followed by several washes. Vessels were then cut open lengthwise and put face down onto MatTek dishes for 30 min in the dark. Images were acquired every 3 sec at 10x magnification and a resolution of 512x512. Stimulation with 3 μmol/L ionomycin resulted in a fluorescence intensity wave traveling along the vessel. Therefore data were analyzed using the area under curve over time over several selected regions of interest selected on the vessels.

Immunoprecipitation and Western Blot

Cells for Western blot analysis were homogenized and resuspended in lysis buffer (50 mmol/L Tris·HCl, pH 7.4/125 mmol/L NaCl/1 mmol/L EDTA/1% NP40/1 mmol/L sodium orthovanadate/20 mmol/L NaF/10mg/ml PMSF/protease inhibitor mixture (Sigma P8340)); for eNOS immunoprecipitation; buffer (50 mmol/L Tris·HCl, pH 7.4/125 mmol/L NaCl/1 mmol/L EDTA/1% NP40/1 mmol/L sodium orthovanadate/20 mmol/L NaF/10mg/ml PMSF/protease inhibitor mixture (Sigma #P8340)). Insoluble material was removed by centrifugation at 12,000 $\times g$ for 10 min at 4°C. Forty micrograms of protein from cell lysates were then analyzed by Western blot analysis, or 500 μg of protein was used for immunoprecipitating eNOS (4 μg/sample mouse anti-eNOS at 4ºC for 2 hrs, Invitrogen #33-4600) followed by protein A – sepharose beads incubation (at 4ºC for 1 hr, Sigma # P9424) and subjected to Western blot analysis. Westerns were probed against total eNOS (pS1177)(Cell Signaling #9574) and Hsp90 (BD Biosciences).

Analysis of Tissue eNOS Expression

Tissue biopsies, obtained from age and sex matched wild type, TSP1 and CD47 null mice were snap frozen in liquid nitrogen and pulverized. Chilled lysis buffer (10 mM HEPES, pH 7.4, 1% lithium dodecyl sulfate (LDS), and 1X phosphatase inhibitor cocktail sets I and II

(Sigma, St. Louis, MO), and 1X protease inhibitor cocktail set I, (Calbiochem, Gibbstown, NJ)) was added to pulverized tissue (20 mg tissue/1 ml buffer) and incubated on ice for 10 min. Each lysate was passed through a 23-gauge needle 10 times, briefly sonicated, and then centrifuged for 10 minutes at 10,000 xg. The supernatants were snap-frozen and stored at -80ºC. Samples normalized by protein concentration (Pierce Micro BCA assay) were mixed with 4X LDS sample buffer containing 2-mercaptoethanol (2% final concentration) and heated for 10 minutes at 70ºC. Tissue lysates (45 µg/lane) were separated by SDS-PAGE (NuPAGE Novex Bis-Tris gels) and transferred onto 0.45 µm Invitrolon polyvinylidine difluoride membranes. After overnight incubation at 4ºC in blocking buffer (7.5% glycine, 5% nonfat dry milk, 0.1% TWEEN 20), the membranes were incubated with a primary rabbit anti-eNOS (clone sc-654, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in wash buffer (25 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.1% TWEEN 20, 0.1% BSA) for 2 hrs at room temperature, washed 5 times (3 min each), and then incubated with diluted secondary antibody (peroxidase (HRP) conjugated goat anti-rabbit (31460), and Alexa Fluor 647 conjugated goat anti-mouse) for 1 hour at room temperature, followed by 5 washes (3 minutes each). Blots were developed with enhanced chemiluminescence reagents (GE Healthcare) and imaged using a Kodak 4000MM imaging system. Alexa Fluor 647 signals were captured with 625 nm excitation and 670 nm emission filters.

Analysis of Tissue sGC Expression

Murine lung tissue (wild type, TSP1 and CD47 null) was harvested and processed with a Dounce homogenizer (1.5 ml) with ice cold RIPA lysis buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mmol/L EDTA, 1 mmol/L PMSF, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, and Roche Complete protease inhibitor (Roche, Indianapolis, IN)). Protein content was determined with the Pierce micro BCA kit (Thermo Scientific Pierce Protein Research Products, Rockford, IL). Cell lysates were subjected to SDS-PAGE on 4-12% gels and the separated proteins were transferred to Immobilon-P transfer membranes (Millipore Corporation, Beford, MA). sGC α1 was detected

with a polyclonal anti-sGC α1 antibody (Abcam, Cambridge, UK), sGC β1 was detected with a polyclonal anti-sGC β1 antibody (Sigma, St. Louis, MO), and actin was detected with a monoclonal anti-α-actin antibody (Calbiochem, San Diego, CA). The blots were developed with an enhanced chemiluminescence substrate (GE Healthcare, Piscataway, NJ) and images captured in a digital format with a Kodak Image Station 4000MM.

***Ex vivo* Arterial Dilation Studies**

Adult male age matched C57BL/6 wild type, TSP1 and CD47 null mice were euthanized by cervical dislocation, the vasculature was gently flushed with warm Krebs buffer via puncture of the left ventricle to remove blood and the thoracic and abdominal aorta cleaned of surrounding connective tissue *in situ* and then dissected free. Myograph incubation buffer containing TSP1 at the indicated concentration was instilled in the vessel lumen, vessels gently occluded and placed in plain buffer for 15 minutes. Following luminal buffer removal segments were ready for myographic analysis. Segments (3 mm in length) were mounted in a dual wire myograph system (Multi Myograph Model 610M). Vessels were allowed to equilibrate in standard Krebs buffer (in mmol/L: 130 NaCl, 14.9 $NaHCO_3$, 5.5 dextrose, 4.7 KCl, 1.17 $MgSO_4$, 1.18 KH_2PO_4, 0.26 EDTA, 1.6 $CaCl_2$, 0.0031 meclofenamate, pH 7.4) and bubbled with 95% O_2 at 37°C. Passive tone of the arteries was normalized to 9.98 mN. Vessels were then allowed to equilibrate until baseline tension remained constant (30-60 min). Vessel viability was confirmed by a contractile response on addition of KCl buffer (in mmol/L: 34.7 NaCl, 100 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 14.9 $NaHCO_3$, 5.5 dextrose, 0.26 EDTA, 1.6 $CaCl_2$, 0.0031 meclofenamate, pH 7.4), repeated twice for 5 min each. Concentration-response curves to phenylephrine were carried out and a dose that produced 80% maximum contraction (EC_{80}) was chosen for establishing vascular tone prior to additional treatments. Vessels achieving at least 65% relaxation to 10^{-6} M ACh were considered to have intact endothelium.

Internal Blood Pressure Measurement via Telemetry

Mice were anesthetized using ketamine and xylazine (90 and 10 mg/kg, respectively). The telemeter catheter was inserted into the left carotid artery and advanced to reach the aortic arch, and the telemeter body (model TA11PA-C20, Data Sciences International, St. Paul, MN) was placed in a subcutaneous pocket. The telemeter signal was processed using a model RPC-1 receiver, a 20-channel data-exchange matrix, APR-1 ambient pressure monitor, and a Dataquest ART 2.3 acquisition system (Data Sciences International). The system was programmed to acquire data for 10 s every 2 min and to calculate 100 min averages of the mean, systolic, and diastolic blood pressure, pulse pressure, heart rate, and activity. Data presented represent the mean ± SD of 8 mice of each strain or treatment group. In experiments were mice received ACh pilot experiments were first performed with a starting dose of 0.1 μg/gram body weight ACh. Finding this dose effectively lowered blood pressure in both wild type and null we then selected a dose that was 20% less based on published results that suggested null mice have enhanced sensitivity to vasoactives reasoning wild type would show minimal response and null would remain sensitive.

Statistics

Unless otherwise indicated all studies were performed a minimum of three times. Statistical significance was calculated with the Student's *t* test or one-way or two-way ANOVA as appropriate with a p value < 0.05 taken as significant using a standard software package (Origin 7, Origin Labs). Comparison of fitted dose-response vasodilator curves was carried out using a two-way ANOVA, followed by a Bonferroni post hoc test where appropriate.

Supplemental Figure Legends

Supplemental Figure 1. TSP1 limits arterial responses by inhibiting endothelial eNOS. Thoracic arterial segments from male C57BL/6 wild type mice were harvested and vasoconstriction to PE ± L-NAME (100 μmol/L) determined (**A**). Thoracic arterial segments from male wild type mice were harvested and vasodilation to the NO donor DETA/NO ± TSP1 (2.2 nM) determined (**B**). Wild type arteries were pre-treated with L-NAME (100 μM) and vasodilation to DETA/NO ± TSP1 (2.2 nM) determined (**C**).

Supplemental Fig. 1