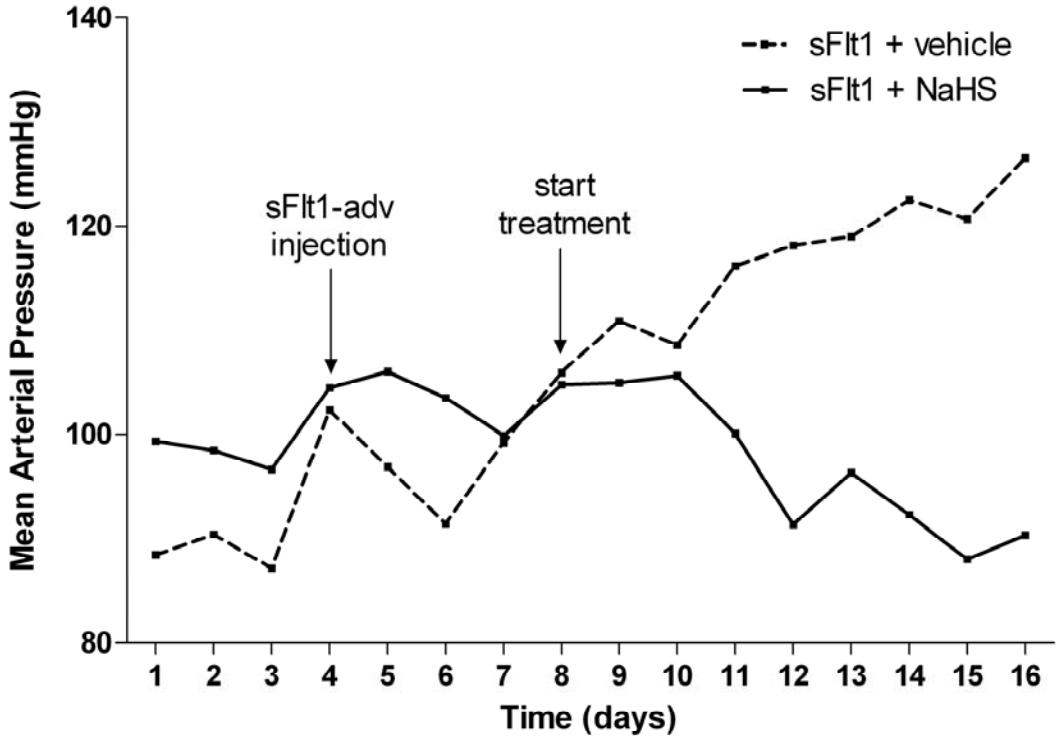
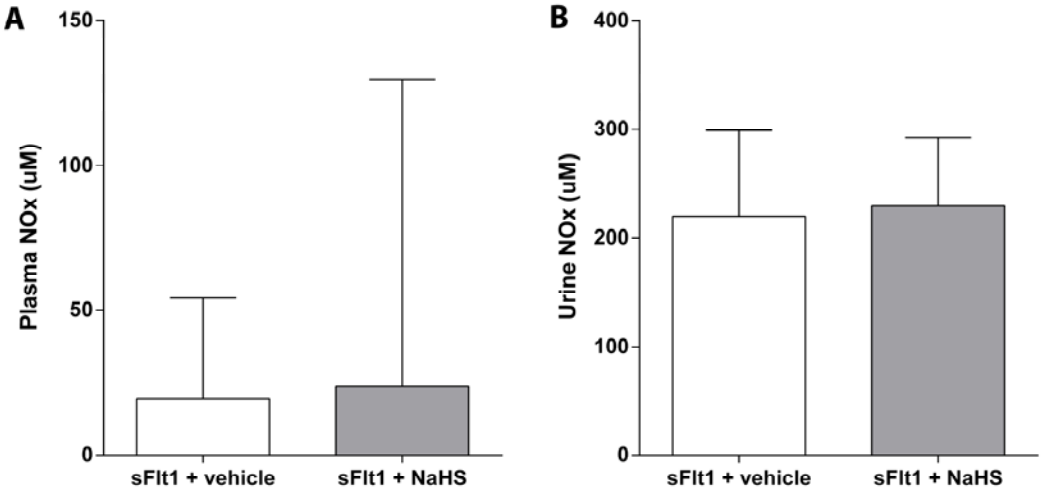
Supplementary Figures

Figure S1 - The effect of NaHS on blood pressure in sFlt1 overexpression rats, measured by telemetry.



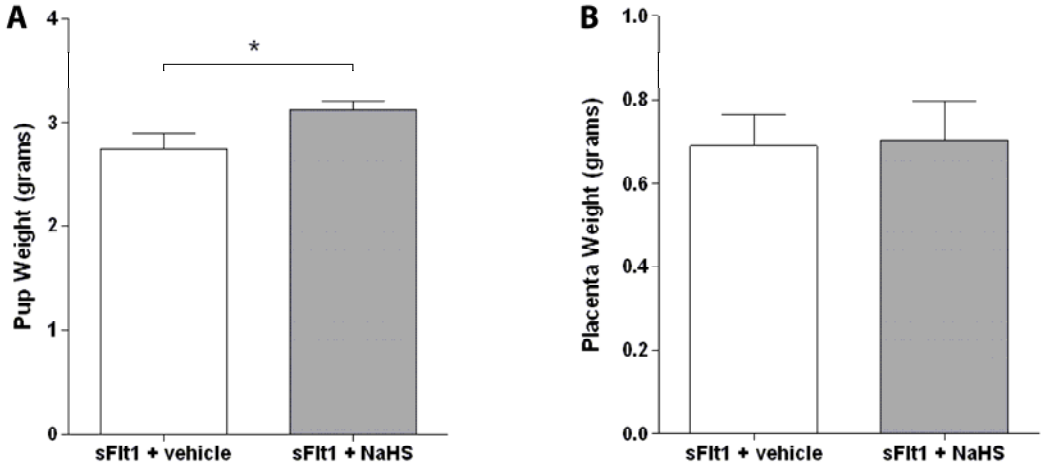
A baseline of the mean arterial pressure was measured during three days. On the day of the sFlt-adv injection, an acute increase in blood pressure is observed (day 4). When treatment with either vehicle (n=1) or NaHS (n=1) started (day 8), the mean arterial pressure increased compared to the baseline. During eight days of treatment, the rat treated with vehicle kept increasing in mean arterial pressure, while the mean arteriel pressure of the NaHS treated rat decreased gradually.

Figure S2 The effect of NaHS on plasma and urine nitric oxide metabolites (NOx).



Data are shown in median (interquartile range). There are no significant differences observed between the NaHS treated and vehicle treated groups, for both plasma NOx (left panel) and urine NOx (right panel).

Figure S3 The effect of NaHS on pup and placental weight.



Data are shown in median (interquartile range). Pup weight in NaHS treated group is significantly ($p < 0.05$) higher compared to the vehicle group (left panel). No differences are observed for placenta weight (right panel).

Supplemental Methods

Experimental design

To analyze the in vivo effect of H_2S on sFlt1 overexpression, rats (n = 14) were treated with either the H_2S donor sodium hydrosulfide (NaHS) or vehicle. The effect of NaHS on proteinuria, hypertension, glomerular endotheliosis and plasma sFlt1 and VEGF levels was evaluated. In addition, to elucidate the mechanism in which NaHS has effects on the sFlt1 phenotype, in vitro studies were performed. The pro-angiogenic effects of NaHS were evaluated by using an endothelial tube assay, the potential of NaHS to up regulate VEGF in vitro was tested in human podocytes, and the vasoactive ability of NaHS was tested by mouse mesenteric vessel myography.

Animal model and NaHS treatment

All animal protocols were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee. Sprague-Dawley female rats (Charles River; 200 – 250 grams) were intravenously injected into the tail vein with $1x10^{10}$ PFU/kg of adenovirus expressing sFlt1. The recombinant adenovirus expressing murine sFlt(1-3) was amplified at a commercial facility (Vector Biolabs, Philadelphia, PA). Plasma sFlt1 levels were determined within 72 hours after adenoviral injection using mouse sFlt1 enzyme-linked immunosorbent assay (ELISA) kit (R&D systems, Minneapolis, Minnesota). Animals with plasma levels of circulating sFlt1 about 500 ng/ml were included in the study and stratified to the treatment (n = 7) or control (n = 7) group. Both groups were treated twice daily for 8 days with either 50 μmol/kg NaHS (Sigma Aldrich, St Louis, MO) or vehicle (PBS), intra-peritoneal.

On the day before the end of the experiment the rats were housed in metabolic cages to collect 24-hour urine samples. Prior to termination and harvesting tissues, blood pressures were measured under anesthesia with isoflurane. The carotid artery was isolated and cannulated with a 3-Fr high fidelity microtip catheter connected to a pressure transducer (Millar Instruments, Houston, Texas). Blood pressure was recorded after normalization and averaged over a 10-minute period. Cardiac puncture for obtain blood samples was performed under deep anesthesia, followed by removal of the kidneys for histopathology.

In a similar design, NaHS was administred to pregnant sFlt1 infected rats. After sFlt1 infection, rats were stratified to treatment and control group and intraperitoneally injected twice daily for 8 days with either 50 μM/kg NaHS (n=6) or PBS (n=6). To assess toxicity of NaHS to pregnancy, pups and placentae were analyzed.

Histopathology and electron microscopy

Harvested kidneys were fixed in 10% formalin, paraffin embedded, sectioned and stained with H&E and periodic acid Schiff (PAS). For electron microscopy, renal tissue was embedded in araldite-epon mixture; 1-µm sections were stained with methylene blue and assessed before ultrastructural study.

Endothelial tube assay

Growth factor reduced BD MatrigelTM (9.0 mg/ml, BD Biosciences, Bedford, MA) was placed into a pre-chilled 96-well culture plate and incubated at 37°C for 30 minutes to allow polymerization. Human umbilical vein endothelial cells (HUVEC) (20,000 cells per 200 µL) were plated onto the MatrigelTM coated wells with or without the presence of 1 µg recombinant sFlt1 (R&D systems, Inc., Minneapolis, MN) and treated with 600 µM NaHS or PBS. After 8 hours, tube formation was assessed through an inverted phase contrast microscope at 4x (Nikon Corporation, Tokyo, Japan) and quantitatively analyzed (number of branching points) using ImageJ software. The experiment was repeated five times.

Stimulation of podocytes with NaHS in vitro

Established lines of human glomerular visceral epithelial cells (GVECs), or podocytes were used. The GVEC culture medium contained 45% Dulbecco's modified Eagle's medium (ICN, Costa Mesa, CA), 45% HAM-F10 (Life Technologies), 1% K1 hormone mix (5mg/ml insulin, 25 ng/ml prostaglandin E1, 0.5 nM T3, 10 nM sodium selenite, 5mg/ml transferrin, 50 nM hydrocortisone (Sigma Aldrich) in Hanks' balanced salt solution), 1% insulin (Sigma Aldrich), and 5% Nu serum (Becton Dickinson, Bedford, MA). Mesangial cell culture medium was composed of 95% Dulbecco's modified Eagle's medium and 5% fetal calf serum (Life Technologies). Culture media were supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin (both from Life Technologies). For the experiment human GVECs were seeded into 12-wells plates and treated with 100 µM or 1000 µM NaHS or PBS. Phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich) was used as a positive control for VEGF up regulation (50 ng/mL). After 30, 60 and 120 minutes, medium was removed and VEGF concentrations were measured using ELISA (R&D Systems). In a parallel experiment, podocytes were harvested and stored for RNA isolation and real-time PCR after 30 min, 2 and 24 hours. All experiments were repeated five times.

Enzyme Linked Immunosorbent Assays (ELISA)

Plasma mouse sFlt1 and rat and human VEGF were measured by ELISA using kits from R&D Systems according to the manufacturer's instructions. The samples were diluted in

0.1% BSA/Tris-buffered saline and were incubated for two hours in a 96-wells plate pre-coated with antibodies against mouse sFlt1 or rat VEGF. The wells were then washed five times in 0.05%Tween 20/PBS and incubated with a secondary antibody against mouse sFlt1 or rat VEGF conjugated to horseradish peroxidase for two or one hour, respectively. Afterwards, the wells were washed five times and a substrate solution containing H2O¬¬2 and tetramethylbenzidine was added. Optical density was determined at 450 nm within 30 minutes. All samples were done in duplicate. For determination of urinary albumin the Nephrat kit from Exocell (Phildadelphia, PA) was used. 24 hour collected urine was diluted 1:10 and incubated with rat albumin conjugate for 30 minutes in a 96-wells plate. After washing six times with tap water a substrate solution containing H2O¬¬2 and tetramethylbenzidine was added. After five minutes, optical density was determined at 450 nm. All samples were done in duplicate.

RNA isolation and real-time RT-PCR

RNA was extracted from snap frozen rat kidney tissue and human GVECs using the TRIZOL method (Invitrogen, Carlsbad, CA). Complementary DNA (cDNA) was synthesized using Superscript II RT and random hexamer primers (Invitrogen). To determine mRNA expression, real-time RT-PCR was performed in a total volume of 20 µL containing 10 ng cDNA and 10 µL PCR-mastermix (Applied Biosystems, Foster City, CA). VEGF-A mRNA was quantified with TaqMan real-time quantitative reverse transcription-PCR, using the VEGF-A gene expression assays (Rn01511605_m1 (rat) and Hs00900055_m1 (human) (Applied Biosystems)). HPRT was used as a housekeeping gene, primer forward: 5'-GGCAGTATAATCCAAAGATGGTCAA-3', primer reverse: 5'-GTCT GGCTTATATCCAACACTTCGT-3' (Invitrogen) and probe: 6-FAM 5'-CAAGCTTGCTGGTGAAAAG GACCCC-3' TAMRA (Eurogentec, Liege, Belgium). The thermal profile was 15 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The average CT values for the target gene VEGF-A was divided by the average housekeeping gene, generating a2-ΔCT value.

Mesenteric vessel wire myograph studies

Rings from second-order mesenteric resistance arteries (MRAs) were harvested from male, wild type C57BL/6 mice and mounted (Danysh MyoTechnology) for isometric tension recordings using PowerLab software (AD Instruments). Briefly, administration of 10 µM phenylephrine (PE) was used to test arterial viability, and the presence of intact endothelium was verified by acetylcholine (Ach, 1 µM)-induced relaxation of a half-maximal PE-induced contraction. Concentration-response relaxation curves were built in the presence of VEGFR2 neutralizing antibody DC101 (50µg/ml) or IgG antibody (50µg/ml) by pre-contracting vessels

with PE at 10 μM before administration of VEGF and NaHS. Data from 2-4 rings per mouse were averaged, with n = 3–4 mice for each wire myograph study.

Implantation of telemetric devices and telemetric data acquisition

Systolic, diastolic and mean arterial pressures were measured using TA11PA-C40 radiotransmitters (Data Sciences International; DSI, St. Paul, MN). For transmitter implantation, female Sprague-Dawlay rats (Charles River; 200 – 250 grams) were anesthetized with isoflurane. The transmitter catheter was secured surgically in the lower abdominal aorta pointing against the blood flow and the transmitter body was placed in the peritoneal cavity, sutured into the musculature of the ventral abdominal wall. Its placement was verified using a radio receiver. Rats were individually housed and recovered for seven days after the surgery. Then, continuous data collection was started using Dataquest A.R.T. Acquisition System.

Measurement of plasma and urine nitrite/nitrate

The stable end products of NO, nitrite and nitrate (NOx) were measured in plasma and urine. As nitrite oxidizes rapidly to nitrate, we used the enzyme nitrate reductase to convert nitrate into nitrite. Samples were diluted fourfold with tap water. NADPH and FAD were added to the samples at a final concentration of 250 μM and 5μM respectively. Subsequently, nitrate reductase (final concentration of 0.2 U/ml) was added to the samples to allow enzymatic conversion of nitrate to nitrite. After incubation for 30 minutes at 37ºC, samples were mixed with lactate dehydrogenase (final concentration of 10 mM) and sodium pyruvate (final concentration of 10 mM). Samples were then incubated for 5 minutes at 37ºC and subsequently 1/20th volume of 30% ZnSO4 was added to the samples. After centrifugation at 13000 rpm for 5 minutes at room temperature, 100 μl supernatant was applied to a microtiter plate well, followed by adding 100 μl Griess reagent (0.01% sulfanilamide, 2.5% phosphoric acid, 0.01% N-naphtyl-ethylene-diamine). After 10 minutes of color development at room temperature, the absorbance was measured on a microplate reader at a wavelength of 540 nm. All samples were done in duplicate.

Statistical analysis

Results are presented as median (interquartile range) OR mean ± S.E.M., comparisons between groups were made using Mann Whitney U test or Wilcoxon signed-rank test, unless otherwise mentioned. Within-group differences (myograph studies) were assessed with two-factor repeated-measures ANOVA with Student-Newman-Keuls post-test. Correlation coefficients were analyzed using linear regression analysis. Spearman correlation

coefficients are presented. All hemodynamic data were analyzed using 24-hr means. Significant differences are reported when $p < 0.05$.