Supplemental Material

Hydrogen Sulfide Attenuates Cardiac Dysfunction Following Heart Failure via Induction of Angiogenesis

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Expanded Methods

Materials and Methods

Mice

Male C57BL/6J mice 8-10 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME). All experimental protocol were approved by the Institute for Animal Care and Use Committee at Emory University School of Medicine and conformed to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996), and with federal and state regulations.

DATS Preparation and administration

DATS (LKT Labs, St. Paul, MN) was maintained in sealed amber glass ampules and kept at -20 °C until use. On the day of experimentation a fresh ampule of DATS was opened. 5 µl of DATS was diluted in 500 µl of 100% DMSO. For *in vivo* experiments, the DATS in 100% DMSO solution was further diluted in sterile saline to obtain the correct dosage to be delivered in a volume of 50 µl. The resulting concentration of DMSO in this dosage was 1%. Vehicle consisted of a solution of 1% DMSO in sterile saline. DATS (200 µg/kg) or Vehicle (1% DMSO) groups were injected intraperitoneally once per day for 12 weeks following TAC.

Transverse Aortic Constriction (TAC) Protocol

To create pressure overload, TAC procedure was performed in mice. Mice were

anesthetized with Ketamine (100 mg/kg) and Xylazine (8 mg/kg) and the core body temperature was maintained in normal range (36-37°C). Then mice were orally intubated and placed on a rodent ventilator to maintain respiration during the surgical procedures. The second intercostal muscle was incised to visualize the aortic arch. Following identification and dissection of the aortic arch, 7-0 silk suture was placed around the aortic arch between the brachiocephalic trunk and the left carotid artery and ligated around a 27G blunt needle. The needle was immediately removed after ligation. The chest was surgically closed and mice were put in a recovery with 100 % oxygen along with a surgical warming pad to maintain core body temperature within normal limits. At the end of the experimental protocol (i.e. 6 or 12 weeks following TAC surgery) mice were euthanized and the hearts, lungs and blood samples were collected, and hearts and lungs were weighed.

Echocardiography

At 2 days prior to TAC procedure, baseline transthoracic echocardiogram was performed using 30-MHz probe on a Vevo 2100 (Visualsonics) under anesthesia with isoflurane (0.25 to 0.50%) supplemented with 100% O_2. Following TAC procedure, echocardiography was also performed in same manner for up to 12 weeks. To determine cardiac structure and function, intraventricular septal end diastolic dimension (IVSd), LV end diastolic dimension (LVEDD), LV end systolic dimension (LVESD), and LV ejection fraction (LVEF) were analyzed from M-mode images.

Histology

Hearts were collected at the indicated times, fixed in 10% buffered formalin, embedded in paraffin stained with Masson's trichrome and Picrosirius Red (to detect fibrosis). Digital images were analyzed using ImageJ.

Vascular density measurements

Angiogenic index measurements from frozen tissue sections were performed as previously described [1,2]. Briefly hearts from the experimental groups were collected, dissected, and embedded in OCT freezing medium. Frozen tissue blocks were cut into 5 µm sections and slides fixed for staining. After FBS blockade, primary anti-CD31 antibody was added to each section at a 1:200 dilution and incubated at 37°C for 1 hour. Slides were then washed with PBS with 1% FBS and subsequently incubated with secondary Cy3 conjugated anti-rat antibody at a 1:250 dilution incubated at room temperature (RT) for 1 hour. Slides were washed with PBS with 1% FBS and mounted with cover slips using Vectashield DAPI. Images were captured using a Hamamatsu digital camera in conjunction with a Nikon TE-2000 epifluorescence microscope (Nikon Corporation, USA) at 200X magnification. Simple PCI software version 6.0 (Compix Inc., Sewickly, PA, USA) was used to measure the area of CD31 and DAPI positive staining to calculate an angiogenesis index expressed as the ratio between CD31 and DAPI positive regions.

We also performed formalin fixed tissue immunohistochemistry using anti-vWF antibody staining to quantify the number of capillaries per unit area. NovaRED staining of vWF was performed using the VECTOR NovaRED peroxidase substrate kit. Briefly, formalin fixed tissue sections were treated with primary and secondary antibodies and NovaRED peroxidase substrate then added for 10 minutes at room temperature in a humidified chamber. After PBS wash, tissue sections were counterstained with hematoxylin. 8 random photomicrograph fields on 2 different tissue sections per mouse were obtained using an Olympus BX53 microscope (Olympus, USA). Capillary density as measured by the number of vWF positive vessels per mm^2 was counted in a blinded manner.

Cellular proliferation measurements

Immunofluorescent staining of the cell proliferation was performed as previously reported [1,2]. Frozen tissue sections of heart were incubated with primary anti-Ki67 antibody (1:350) at 37^0C for 1. Slides were washed with PBS with 1% FBS, and secondary DTAF anti-rabbit (1:150) conjugated antibody was added and incubated at room temperature for another 1 hour. Slides were finally washed and mounted with cover slips using Vectashield DAPI. Images were acquired as described above. Cellular proliferation (proliferation index) was determined as the ratio between regions positive for Ki67 and DAPI positive areas.

Western Blot Analysis

Myocardial tissue samples were taken homogenized and lysates were used for Western blot analysis. Protein concentrations were measured with the DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were loaded into lanes of polyacrylamide-SDS gels. The gels were electrophoresed, followed by transfer of the protein to a PVDF membrane. The membrane was then blocked and probed with primary antibodies overnight at 4°C. The following primary antibodies were used: CD 31 (1:2,000 Abcam) VEGF-A (1:25,000 Cell Signaling); b-FGF (1:2,000 Cell Signaling); Angiostatin (1:3,000 Abcam); Phosphorylated Akt (Ser 473) (1:5,000, Cell Signaling Technology); AMPK and Phosphorylated AMPK (Thr 172) (1:5,000, Cell Signaling Technology); eNOS (1:5,000, BD Transduction Laboratories); Phosphorylated eNOS (Ser 1177) (1:1,000 Cell Signaling Technology); Phosphorylated eNOS (Thr 495) (1:1,000 Cell Signaling Technology); GCLC (1:3,000 Abcam); HO1 (1:10,000 Abcam) Immunoblots were next processed with the appropriate secondary antibodies (Cell Signaling) for 1 hr at room temperature. Immunoblots were then probed with a SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) to visualize signal, followed by exposure to X-ray film.

Measurement of Nitrite Levels

Nitrite analysis was performed as previously described.[3]

Measurement of Hydrogen Sulfide and Sulfane Sulfur

Hydrogen sulfide and sulfane sulfur levels were measured in heart and blood according to previously described methods [4]. For heart tissue, the amount of H_2S is reported as nmol/mg wet weight. For the blood, the amount of H_2S is reported as μM.

Statistical analysis.

All data are expressed as mean ± SEM. Statistical significance was evaluated using unpaired Student t-test for comparison between 2 means and a 1-way ANOVA with a Tukey test as the posthoc analysis for comparison among 3 or more means by use of Prism 5 (GraphPad Software Inc). For the echocardiography data, a 2-way repeated measures ANOVA with a Bonferroni test as the posthoc analysis was used. The following comparisons were made separately: (1) baseline vs. post-baseline measurements at each time point for the DATS and vehicle groups and (2) DATS vs. vehicle measurements at each time point. The p-value for these evaluations was adjusted by applying the Bonferroni correction for multiple comparisons. A value of $p<0.05$ denoted statistical significance and p-values were two-sided.

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