Aortic Remodeling Following Transverse Aortic Constriction in Mice is Attenuated with AT_1 Receptor Blockade

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Materials and Methods

Mice

Twelve week old male C57BL/6 mice were underwent TAC using a standard surgical protocol. [1] Studies were performed 2 weeks after TAC, prior to the maladaptive cardiac failure associated with TAC. [1] Animals were cared for according to the NIH Guide for the Care and Use of Laboratory Animals. All animal experiments were performed under protocols approved by the University of Texas Health Science Center at Houston in accordance with NIH guidelines.

Transverse Aortic Constriction (TAC)

All mouse experimental procedures were approved by the Institutional Animal Care and Use Committee of Medical Animal Resource Center, University of Texas Health Science Center at Houston. C57BL/6 male mice (10–12 weeks of age, 18–23 g, Jackson Laboratories, Bar Harbor, ME, USA) will be used in the surgical preparation. Prior to anesthesia each animal will receive a dosage of buprenorphine (0.1-2.5 mg/kg subcutaneous injection). The animal will then be anesthetized using 2% isoflurane in 0.5 -1.0 L/min 100% oxygen. The neck and chest areas will be prepared by shaving and removing hair, cleansing the skin with surgical soap followed by wiping with 70% ethanol. This procedure is repeated three times. Prior to surgery, all instruments are sterilized in a dry bead sterilizer. The anesthetized animal is placed in a supine position and a 5mm section of the trachea is carefully exposed by mid-neck incision and retraction of muscle tissue. This allows visualization for insertion of the endotracheal tube which is a polyethylene size 90 tubing beveled on the edge for ease of entrance through the larynx. The tongue is carefully manipulated as the endotracheal tube is inserted into the trachea with visibility through a dissecting microscope, viewing the trachea and entrance of the endotracheal tube. Once the proper position is confirmed, the endotracheal cannula is connected to a volume-cycled rodent ventilator (CWE, Inc.) which runs on supplemental 100% oxygen with a tidal volume approximately 0.15-0.25ml and a respiratory rate of 100-125 breaths per minute. Once steady breathing is established, an incision is made through the ventral chest skin to mid-thorax after which the thorax is opened to mid-sternum. This partial thoracotomy is followed by retracting the sternal edges with a retractor (fine science tools). The thymus is then retracted to expose the transverse aorta. Between the right innominate and left carotid artery, an aortic constriction is placed by tying a 6-0 suture black braided nonabsorbable silk suture) against a 3mm length of 27 gauge needle. After two knots, the 27 gauge needle is promptly removed which yields a constriction of approximately 0.3mm as the outer diameter of the 27 gauge needle. This produces a 60-80% aortic constriction which can be double checked using the doppler flow option on the Vevo ultrasound machine. The outflow is then briefly (1-2s) pinched off on the respirator to allow re-inflation of the lungs. The retractor is removed and the ribs are drawn together and sutured using 5-0 Vicryl suture. Once the chest is closed, the outflow is briefly pinched off again to ensure proper breathing. The skin is then closed using 5-0 non-

absorbable sutures, which will be removed within 10 days post-surgery. Once all sutures are in place, anesthesia is stopped and the animal is allowed to recover and will be removed from the ventilator. The sham group mice were performed the same operation but did not ligate the aorta. The animal will be monitored closely for any abnormal signs of pain or labored breathing before being returned to the animal room. In the case of any signs of pain, the animal will receive another dosage of buprenorphine (0.1-2.5 mg/kg subcutaneous injection) every 6-12 hours when needed.

Mouse Doppler echocardiography procedure

The successful TAC procedure was validated by Doppler Imaging. Specifically, the TAC procedure, which produces a 60-80% aortic constriction, was monitored for such constriction by a specially designed non-invasive Doppler flow probe. The flow characteristics indicate the magnitude of constriction; can be easily accessed by measuring the pulsatility index of flow in each of the carotid arteries, post-aortic constriction. This non-invasive Doppler procedure was performed 1 and 2 weeks postTAC procedure. Only mice with a right carotid (RC)/left carotid (LC) flow ratio within a certain range were included for further analysis. For example, a moderate degree of pressure overload leads to a ratio of 5-8, whereas a tighter constriction resulting in severe pressure overload leads to a ratio of 8-10. A sham animal (operated but not ligated), however, is expected to have a ratio of ~1.Therefore, RC to LC flow velocity ratio reached a gradient greater than 5-10-fold higher by Doppler indicated a successful transverse constriction [1].

High resolution ultrasound was performed with a Vevo 770 imaging system using a 40 MHz 704 probe (VisualSonics, Toronto, Canada). Mice were anesthetized by inhalation of 1.5-4% isoflurane-oxygen mixture while echocardiography and respirations were continuously monitored on a warmed platform. The anterior chest was denuded using depilatory cream prior to application of ultrasound gel and imaging. The dimensions of the ascending aorta (in mm) were measured in a single plane using right parasternal views at four levels (annulus, sinuses of Valsalva, sinotubular junction and ascending aorta). The flow velocities in the aorta were recorded. After stabilization of the signal (1 min), peak systolic velocity (V, cm/sec) was recorded as the mean of three to five cardiac cycles. Images were then exported to Sante DICOM Editor software (SanteSoft LTD, Athens Greece) for further analysis. In all studies, at least five animals per group were used. All scanning and analysis was performed by an experienced ultrasound technician who was blinded to the mouse genotype.

Histomorphometric study

After Doppler study confirmed increase pressures and echocardiography assessed the ascending aortic diameter, animals were anesthetized and perfusion fixed with 5 ml of 1XPBS, followed by 5 ml of 4% paraformaldehyde for 3 min under physiological pressure. Ascending aorta, descending aorta, left and right carotid arteries were excised and further fixed overnight. The tissues were then embedded transversely in paraffin and cut at 5 μm thickness. Cross-sections were stained by haematoxylin and eosin (H&E) following standard protocol. For morphometric analyses, images of H&E stained cross-sections were be taken and recorded with a camera connected to a light microscope at 10x and 40X magnifications. The luminal, medial and adventitia areas of each cross-section were analyzed using Image J software. The medial area was defined as the area between the external and internal elastic lamina. Because the intimal thickness was not increased, the luminal area was the same as the internal elastic lamina area. The adventitia area was defined as the area between the external elastic lamina and the tunica externa, the outermost layer of the vessel. Sections were also stained with Sirius red to determine the collagen content and with Verhoeff-van Gieson elastin staining to detect the expression of elastin. For all these area measurements, two sections from a sample from one mouse were chosen at random. Five mice for each time point were analyzed and averaged.

Immunohistochemistry and immunofluorescence
Immunohistochemical staining was performed on paraffin-embedded sections with primary antibodies against Phospho-Histone H3 (PH3, Upstate) and Smooth muscle-α actin (α-SMA, Sigma-Aldrich), followed by biotinylated secondary antibody according to the manufacturer's protocol. Staining was treated with peroxidase-conjugated biotin–avidin complex using VECTASTAIN ABC-AP kit (Vector Laboratories) and visualized by DAB (Vector Laboratories). Slides were counterstained with hematoxylin. For immunofluorescence staining, slides containing frozen mouse aortic cross sections (6-μm) were immediately fixed with 4% paraformaldehyde for 30 minutes, blocked using 0.1% Triton-X, 5% normal serum of the species producing the highly cross-absorbed Alexa Fluor 568–conjugated secondaryantibody (Invitrogen) for 30 minutes at 37°C and then incubated with primary antibodies at the following concentrations: 1:100 anti-CD68 (eBioscience), 1:100 anti-fibroblast ER-TR7(Bachem), 1:50 anti–MCP-1 (ECE.2; Abcam), 1:200 anti–IL-6 (eBioscience), and 1:200 anti-macrophage MOMA-2 (Abcam). Incubations were performed for overnight at 4°C. After washing, secondary antibodies were added at a dilution of 1:200 for 1 hour at 37°C. Slides were then DAPI stained (Vector Laboratories).

***In situ* DHE oxidation**
Aortas from sham- or TAC mice were dissected, OCT embedded, and frozen in a methybutane/ethanol/dry ice bath. Frozen sections (8-μm) were mounted on glass slides, rinsed in PBS, and incubated in 10 μm DHE (37°C, 30 minutes; Invitrogen). Then slides were DAPI stained, mounted, and photographed using a Texas red filter (488-nm excitation, 610-nm emission).

RNA Extraction and Quantitative Real-time PCR
RNA was extracted from each mouse ascending aortic tissue using Trizol (Invitrogen) according to the manufacturer's protocol. 0.5 μg RNA was reverse transcribed using the High Capacity cDNA Archive Kit (Life Technologies) according to the manufacturer's protocol. For quantitative real-time PCR analysis of mRNA expression, TaqMan probes were purchased from Applied Biosystems and analyzed using an Applied Biosystems Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Experiments were performed in triplicate. *Gapdh* was used as the endogenous control.

Immunoblot analyses
Protein lysates were prepared from each mouse aortic tissue following standard protocol. Briefly, aortic tissues were homogenized and lysed in RIPA buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Protein (25μg) for each sample was separated on Tris–HCl gel (Bio-Rad, Hercules, CA) by SDS-PAGE, followed by transfer to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were immunoblotted with primary antibody and the appropriate horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunoblots blots were visualized by the enhanced chemiluminescence technique (GE Healthcare, Piscataway, NJ). The following dilution of antibody was used: total ERK (cell Signaling), 1:1000; anti-phosphorylated ERK (cell Signaling), 1:1000; anti-phosphorylated Smad2 (cell Signaling), 1:1000; anti-phosphorylated Smad3 (cell Signaling), 1:1000; anti-GAPDH (Fitzgerald Industries, Acton, MA), 1:2000.

Losartan treatment

Losartan Potassium (sc-204796A, Santa Cruz) was administered by oral at a dose of 0.6g/l in drinking water (n=6) for 3 days prior to TAC. Mice were continued on oral therapy for 2 wks after TAC and then sacrificed.

Statistical analysis

Data are expressed as the means ± standard deviation. Statistical differences between the data at different time point were analyzed by a Student's t-test. Morphometric analysis of aorta and carotid artery was done by one-way ANOVA. Differences were considered statistically significant at values of $P < 0.05$.

References

(1) deAlmeida AC, van Oort RJ, Wehrens XH. Transverse aortic constriction in mice. *J Vis Exp.* 2010;38:1729