Smooth Muscle Cell Deletion of LDL Receptor Related Protein-1 Augments AngII-Induced Superior Mesenteric Arterial and Ascending Aortic Aneurysms

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MATERIALS AND METHODS

Mice and diet

The LRP1flox mouse was developed using cre-mediated recombination resulting in a deletion of a portion of the LRP1 promoter and transcription site, as well as exons 1 (signal peptide coding region) and 2.[1] A search in the Entrez data base for miRNA expressed in the mouse LRP1 gene revealed no known miRNA present. Smooth muscle-specific LRP1 deletion was accomplished by breeding SM22 Cre transgenic mice to LRP1 floxed mice.[2] Male smLRP1-/- and littermate +/+ mice in a mixed background were separated into saline and AngII infusion groups.[2] Mice were housed 5 per cage in individually ventilated cages with negative air pressure (Allentown, Inc; Allentown, NJ). Aspen hardwood chips (Sani-Chip; Cat # 7090A; Harlan; Madison, WI) were used as bedding. Drinking water was filtered by reverse osmosis (pH 6.0-6.2). Light cycle of the room was 14 hours of light and 10 hours of dark. Ambient temperature ranged from 68 to 74˚F and humidity was 50-60%. All mice were 6-8 weeks old at the start of study and were fed normal laboratory mouse diet (Cat # 2918; Harlan Teklad Global 18% Protein Rodent Diet; Madison, WI). Necropsy was performed to determine cause of death during infusion. Genotypes were confirmed at termination using DNA isolated from tails and PCR using Cre primers. All procedures were approved by the University of Kentucky IACUC.

Infusions

Saline, AngII and NE were infused via subcutaneous pumps as described previously.[3,4] Osmotic mini-pumps (Alzet Model #2004; Durect; Cupertino, CA) were filled with saline, AngII (1,000 ng/kg/min dissolved in saline) or NE (5.6 mg/kg/day; dissolved in saline containing 0.2% ascorbic acid).[4,5] Mice were sedated with Isoflurane (Isothesia; Butler Animal Health Supply; Dublin, OH) and pumps were implanted subcutaneously on the right side of the mice. Surgical staples were used to close incision sites and a topical anesthetic cream (LMX4; Ferndale Laboratories; Ferndale, MI) was applied.

Ultrasonic imaging

Ascending and suprarenal regions of aortas were imaged *in vivo* using a Vevo 2100 with a MicroScan MS400 transducer (18-38 MHz, resolution 30 µm; VisualSonics; Toronto, Ontario, Canada).[6,7] Mice were anesthetized using Isoflurane (Isothesia; Butler Animal Health Supply; Dublin, OH) and hair was removed from the chest and belly using a hair clipper and depilatory cream (Nair; Church and Dwight Co; Princeton, NJ). Ultrasonic gel (Medline; Mundelein, IL) was placed on the shaved areas and a transducer was used to capture images. B-Mode ultrasonic images were measured to obtain the maximum systolic diameter of ascending and suprarenal regions of the aorta. A mean of three cardiac cycles was used for diameter measurements in each mouse.

Systolic blood pressure measurements

A non-invasive tail cuff system (Kent Scientific; Torrington, CT) was used to measure systolic blood pressure at baseline and during week 3 of infusion. Conscious mice were restrained on a heated platform. Blood pressure was measured the same

time each day for at least three consecutive days. Accepted data criteria were 10 of 20 measurements (50%) and a SD <30.[8]

Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) was performed using a 7.0-T Clinscan system (Bruker, Ettlingen, Germany) with a 30 cm inner diameter quadrature radiofrequency coil.[9] Mice were anesthetized with Desflurane and positioned in the supine position in the imaging unit. Body temperature was maintained at 36.5 ± 0.5 °C using a heated water circuit. Electrocardiographic data, body temperature and respiration rate were continuously monitored throughout the scan using an MRI-compatible system (SA Instruments, Stony Brook, NY).

To span the length of the aorta from top of the arch to iliac bifurcation, two transverse stacks of cardiorespiratory-gated gradient echo bright blood images were acquired with in-plane resolution of 0.1198 x 0.1198 mm^2, slice thickness of 0.6 mm, no slice gaps, and 14-15 phases per cardiac cycle. In each case, the thoracic stack (43 slices) was acquired first, then the mouse bed was manually re-positioned with respect to the center of the cylinder coil, followed by acquisition of an abdominal stack (35-40 slices). Four fiducial markers (100 IU vitamin E tablets) were aligned and positioned under the mouse serving as reference points between the two imaging positions to ensure overlap between the slice stacks. Separate respiratory-gated, T1-weighted sagittal localizer stacks were acquired to visualize and define positions of these markers in both bed positions. Additional gradient echo images were acquired in oblique views of the aortic arch for qualitative assessment.

MRI Post-processing

Transverse image stacks were interpolated to obtain an approximately isotropic 3-dimensional image volume.[10] The aorta (inclusive of the takeoffs of the head and neck vessels, the superior mesenteric artery takeoff and the iliac bifurcation) was semi-automatically segmented in a single cardiac phase using custom software written in MATLAB (The Mathworks, Natick, MA). To merge segmented aortic masks, one set of fiducial marker images was separately segmented with a 3D level set segmentation algorithm. Translation of a single marker between positions was determined based on visual registration of the 3D segmentation mask with the corresponding marker in the second localizer stack. The resulting translation was imposed on the 3D image position coordinates for the appropriate transverse image stacks. Additional small adjustments to the registration were made in some cases to maintain consistency with anatomic landmarks. Once merged, the segmented isosurface was defined and complete reconstruction was exported for geometric feature extraction.

Geometric analysis was performed using the Vascular Modeling Toolkit (VMTK; vmtk.org), with results visualized in ParaView (paraview.org). The 3D surface was first smoothed for 30 iterations with a passband of 0.1, and the vessel centerline calculated and smoothed (100 iterations; smoothing factor 0.1). From this centerline, maximum and average radii of the aorta and superior mesenteric arteries (based on the maximum inscribed sphere radius at each point along the centerline), length of the aorta from left

subclavian artery takeoff to iliac bifurcation, and aortic tortuosity from left subclavian artery takeoff to iliac bifurcation were calculated. Tortuosity (T) was defined as the ratio of the centerline distance traveled versus the straight line distance connecting the two end points.

Isolation and culture of vascular SMCs

Mice were euthanized with a ketamine/xylazine solution (90/10 mg/kg, respectively; Henry Schein, Oshkosh, WI). Arterial vasculature was perfused through the left ventricle of the heart with sterile PBS. Aortas were excised, and dissected free of adventitia and fat. Aortas were separated into thoracic (from the aortic valve to the last intercostal) and abdominal aortic regions (from the last intercostal to the bifurcation). Vascular SMCs were isolated from aortas as described previously.[11,12] SMCs from 4 pooled mice were plated 1 well of a 6 well plate and grown in DMEM, FBS(20%) and penicillin and streptomycin (1%) and placed in a 5% CO_2, 37^{o}C incubator. Vascular SMC phenotype was determined by immunostaining cells for α-actin (Abcam, ab5694, Cambridge, MA). Cells were grown to 80-90% confluency and serum-starved for 24 hours before use.

Western blot analyses

Aortic tissue homogenate or total cell lysates of primary vascular SMCs harvested from either thoracic or abdominal aortic regions were prepared in RIPA lysis buffer. Protein concentration was measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein samples (2.5-10 g) were resolved by 4-20% SDS-PAGE and transferred electrophoretically to PVDF membranes. After blocking, antibodies against the following proteins were used to probe the membranes: LRP1 (Abcam, ab92544, Cambridge, MA) and β-actin (Catalog# A5441, Sigma-Aldrich, St. Louis, MO). Then membranes were incubated with either biotinylated goat anti-rabbit or biotinylated rabbit anti-rat secondary antibodies (Cat # T1101 and T10813, Vector Laboratories; Burlingame, CA), and immune complexes were visualized by chemiluminescence (Catalog number 34080; Thermo Scientific, Waltham, MA) and quantified using a Thermo Scientific myECL Imager.

Pathology

At experimental termination, mice were anesthetized with an i.p. injection of ketamine/xylazine (90/10 mg/kg, respectively; Henry Schein, Oshkosh, WI). Blood was collected by cardiac puncture, and aortas were flushed with saline. For the measurement of length and *ex vivo* photographs, aortas with hearts and kidneys were dissected free and tissues were immersion fixed overnight in 10% formalin. Extraneous tissue was removed and ascending aortas were photographed. The regional length of the aorta in either the thoracic + suprarenal region (from left subclavian artery to left renal artery) or the infrarenal region (from the left renal artery to bifurcation) was measured *ex vivo* using Image Pro Version 7.0 software. For immunostaining and pathology, aortas and SMAs were dissected free and extraneous tissue was removed. Ascending aortas and SMAs were placed in OCT without fixation.

Ascending aortas and SMAs were serially sectioned (10 μm thickness; multiple series of ten slides with 9 sections/slide). H&E and Movat's pentachrome stains were used to examine cellularity and elastin fibers. Immunostaining was performed to detect specific cell types. SMAs were quantified using artery cross sections that were stained with H&E at 0.9mm-1.8mm from the bifurcation of the SMA and aorta. Anterior-posterior (AP) and transverse (trans) diameters were measured on 9 serial 10 μm sections per mouse. A mean was determined for measurements from these 9 serial sections to give a SMA diameter for each mouse. For immunostaining, the following antibodies were used: α-actin for smooth muscle cells (Cat # ab5694; Abcam; Cambridge, MA) and CD68 for macrophages (Cat# MCA1957GA, Serotec; Raleigh, NC). Reactivity was determined using biotin labeled secondary antibodies (Cat # BA-4001 and BA-6000; Vector Laboratories; Burlingame, CA), horseradish peroxidase ABC kits (Peroxidase Elite Standard; Cat # PK-6100; Vector Laboratories; Burlingame, CA) and 3-amino-9- ethylcarbazole for chromogen (ImmPACT™; Cat # SK-4205; Vector Laboratories; Burlingame, CA). Non-immune primary antibodies, secondary only, and no primary or secondary antibody slides were run as negative controls to confirm specificity of primary and secondary antibodies, and ablation of endogenous tissue peroxidase, respectively.[13] Elastin breaks were counted at the maximum ascending aortic or superior mesenteric artery expansion. For each mouse, fragmentation was counted in 5 serial 10 μm sections and then meaned. Four mice were used per group, and group mean elastin fragmentation was reported.

Real Time PCR

Ascending aortas were dissected free and adventitia left intact. Total RNA was extracted from ascending aortas using the Qiagen Rneasy Fibrous Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Reverse transcription and real time PCR were performed using an iCycler (Bio-Rad).[13] RNA (100 ng) was reverse transcribed using the i-script cDNA synthesis kit (Bio-Rad). PCR product accumulation was monitored using a custom designed 96-well Taqman gene expression assay plate according to manufacturer's instructions (Life Sciences, Grand Island, NY). The gene expression assays included on the custom 96-well plates are in Supplemental Table III. mRNA abundance was calculated using the ΔΔCt method with normalization to either β-actin or GADPH.

Statistical analyses

Statistical tests included standard parametric procedures for a single response and repeated measures. Analyses were performed using SigmaPlot version 12 (Systat Software Inc, San Jose, CA). The specific statistical test used is identified in each figure legend. Holm-Sidak for post hoc testing was used to ascertain group differences in two-way ANOVA. Data are represented as individual and mean ± SEM. $P<0.05$ was considered statistically significant. All positive phenotypes attained a power of greater than 0.8.

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