MATERIAL AND METHODS

Detailed Methods

Mice. All studies using mice were approved by the institutional Animal Care and Use Committee at the University of Kentucky and conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH. Male transgenic mice with deletion of *Sry* from the Y-chromosome expressing *Sry* on an autosome (8-12 weeks of age) were backcrossed 10 times on a C57BL/6J background (Stock#010905, The Jackson Laboratories, Bar Harbor MA) and bred to low density liproprotein receptor deficient (*Ldlr*$^{-/-}$) females (Stock# 002207, The Jackson Laboratories, Bar Harbor MA) to generate *Ldlr*$^{-/-}$ male mice with an XY or an XX sex chromosome complement. DNA was extracted from ear or tail clips and subjected to PCR using a commercial PCR mix (Promega 2X Master Mix, cat#m7123, Fitchburg WI) and specific primers. For transcriptome analysis, male XY and XX *Ldlr*$^{-/-}$ mice (n = 4-5/group/genotype) were randomly assigned to sham and orchiectomized (ORC) groups. Following surgery, mice were fed a Western diet (TD88137, Harlan Teklad, Indianapolis, IN) for 2 weeks prior to euthanasia and tissue harvest. For assessment of aortic vascular pathologies (Supplemental Figure IA), male XY and XX mice (n = 19-21/group/genotype) bred from transgenic male *Sry* mice were randomly assigned to sham and ORC groups. In addition, we included XY male *Ldlr*$^{-/-}$ mice (n = 10) purchased from The Jackson Laboratories (Stock# 002207, Bar Harbor MA) to compare to XY males generated from our transgenic *Sry Ldlr*$^{-/-}$ colony. As there were no observed differences in data quantified from *Ldlr*$^{-/-}$ XY males (*Sry*-bred or from The Jackson Laboratory), data from XY males were pooled between strain sources. Two weeks after surgery, mice were fed the Western diet for 1 week prior to implantation of osmotic minipumps and then through study endpoint. Anesthetized mice were implanted subcutaneously with osmotic minipumps (Alzet model 1004, Durect Co., Cupertino, CA) for infusion of AngII for 28 days (1,000 ng/kg/min, Bachem, Torrance, CA). Ultrasound was performed on anesthetized mice on day 0, 7, 14 and 27 of AngII infusions. In this study, n = 6 sham-operated male XX mice, n = 2 ORC male XX mice, n = 2 sham-operated male XY mice and n = 2 ORC male XY mice died from aneurysm rupture, and thus were not included in some AAA quantification measurements (internal aortic diameters, external AAA diameter). For aortic regional mRNA abundance, male XY and XX *Ldlr*$^{-/-}$ mice (n = 6-8 mice/sex) were infused with saline or AngII (1,000 ng/kg/min) for 1 day prior to euthanasia and tissue harvest. For prolonged infusion of AngII (Supplemental Figure IB), male XY and XX *Ldlr*$^{-/-}$ mice (n = 19 XY, n = 8 XX) were fed the Western diet for one week prior to implantation of osmotic minipumps containing AngII (1,000ng/kg/min) and the diet continued through study endpoint. Ultrasound was used on day 27 of AngII infusions to define mice with an AAA (abdominal aortic lumen diameter > 50% increase compared to day 0 of infusions). For each genotype, all mice infused with AngII qualified with an AAA and thus continued in the study. Osmotic minipumps were replaced with fresh AngII solution every 28 days in anesthetized mice. Ultrasound was performed on anesthetized mice on day 0, 14, 27, 42, 56 and 70 of prolonged AngII infusions. In this study, n = 9 male XY mice and n = 3 male XX mice died from AAA rupture and thus were not included in some measures of AAA quantification (e.g., ultrasound or maximal AAA diameters). Thus, illustrated data for these measurements represent mice that survived the 70 days of AngII infusions. At the end of each experiment, mice were euthanatized under anesthesia (ketamine/xylazine, 100:10 mg/kg, i.p.) and blood was collected via cardiac puncture.

Orchiectomy. Male (8-12 weeks of age) XX and XY *Ldlr*$^{-/-}$ mice were orchiectomized under isoflurane anesthesia (2-3%) and given pre and post-operative analgesic (24 hr after surgery; flunixin; 2.5 mg/kg). Mice were shaved in the scrotum region and a depilatory cream (Nair, Inc.) applied to the area to remove hair, followed by sterilizing with povidone-iodine/ethanol (three times). A small incision was made in this region. Vas deferens were collapsed using a hemostat and the testes were removed. The vascular supply was ligated by cauterization using a high-

temperature fine-tip loop cauterizer and the hemostat was released. The wound site was monitored for bleeding, and the skin was closed by wound clips (Autoclip stapler). The site was then treated with povidone-iodine, and mice were allowed to recover. The testes were manipulated but left intact in anesthetized sham-operated mice. All mice were allowed to recover from surgery and to clear their endogenous testicular hormones for two weeks before the onset of AngII infusions.

Measurement of plasma and serum components. Total serum cholesterol and testosterone concentrations were determined using enzymatic assay kits (Wako Pure Chemical, Richmond, VA, cat#439-17501; Alpco, Salem, NH, cat#55-TESMS-E01; respectively). The renin concentration in plasma was measured by quantifying angiotensin I generated in the absence or presence of an excess of exogenous rat angiotensinogen (purified from nephrectomized rat plasma). Plasma was harvested from mice in ice-cold EDTA (0.2 M). Mouse plasma (8 µl) was incubated in buffer (Na_2HPO_4, 0.1M; EDTA, 0.02 M; maleate buffer, pH 6.5; total volume of 250 µl) containing phenylmethylsulfonyl fluoride (2 µl/250µl reaction volume) for 30 minutes at 37°C in a shaking water bath. The reaction was terminated by placing samples at 100°C for 5 min. Angiotensin I was quantified by radioimmunoassay using a commercial kit (DiaSorin, CA-1553, Stillwater, MN). Plasma renin concentration (ng/ml) is represented as the difference in angiotensin I levels in the absence versus the presence of exogenous angiotensinogen.

Blood pressure measurements. Blood pressure was quantified using a non-invasive computerized tail-cuff method (BP-2000; Visitech Systems, Apex, NC) at baseline (prior to pump implantation) and during week 3 of AngII infusion. Measurements were recorded at the same time of the day for 5 days (2 days acclimation, 3 days of recording).

Quantification of AAAs. The abdominal aortic internal lumen diameters were quantified on day 0, 7, 14, and 28 of AngII infusions using a Vevo 2100 high-resolution imaging system (VisualSonics, Inc.). AAA incidence was quantified from the abdominal aortic internal lumen diameter, and defined as a 50% increase in lumen diameter on day 28 of AngII infusions (compared to day 0). AAA incidence included mice that died from aneurysm rupture, which was visually confirmed post-mortem. Other measurements (ultrasound, maximal AAA diameters) included mice that survived the complete study protocol. External abdominal aortic diameters were quantified on excised, cleaned tissue mounted on a black wax background. A Nikon SMZ800 dissecting microscope was used to obtain aorta images and the maximal external diameter of the abdominal aorta was quantified. Using the Vevo 2100 system with a 55-MHz probe, the three-dimensional volume analysis was performed on abdominal aortas of XX and XY males (n = 5 mice/genotype) that were infused with AngII for 3 months. Aortas were scanned at the abdominal region (diaphragm to the ileac bifurcation) using the ultrasound ex vivo at 0.051 mm intervals with an imaging length of 14.986 mm, and a total of 295 images were collected. Each image was analyzed for wall and lumen volume, and images were combined to obtain the total volume of abdominal aortas.

Quantification of AAs and TAAs. Arch areas were defined by drawing a 3-mm line from the left subclavian artery. The intimal area of the aortic arch was defined as the region from the orifice of innominate artery to the orifice of the left subclavian artery. Thoracic areas were defined by drawing a 9mm line from the end of the arch area to the diaphragm muscle. Arch and thoracic combined areas were quantified by drawing a line around the borders and summing the total area of each. Measurements were performed using Nikon Elements Version 3.2.

Quantification of atherosclerosis. Atherosclerosis was quantified en face in the aortic arch.[1] Lesions were summed and divided by the total arch area to calculate the percent lesion area. Cleaned aortas were cut open longitudinally and mounted on a black wax background using

insect pins (Fine Science Tools, cat# 26002-20) to quantify atherosclerosis in the aortic arch and the thoracic aorta.

Quantification of aortic stiffness (pulse wave velocity, PWV). Aortic stiffness was assessed on anesthetized (2% isoflurane) male XY and XX mice (8 months of age, non-infused, n = 6 mice/genotype) that were placed supine on a heating board with legs secured to ECG electrodes.[2] Doppler probes (Indus Instruments, Webster, TX) were used to noninvasively quantify aortic velocities at the aortic arch and abdominal aorta. PWV was calculated as the distance between the aortic arch and abdominal probes divided by the difference in thoracic and abdominal aortic pre-ejection times. Data are presented as centimeters/second.

Quantification of adventitial and medial diameter in thoracic aorta tissue sections. Thoracic aortic segments from XY and XX male mice (n = 3 mice/ group) infused with AngII for 28 days were formalin fixed, processed through a series of ethanol and xylene baths and embedded in paraffin. Sets of serial 5 micron cross sections were collected at intervals along each segment and stained with hematoxylin and eosin. Six tissue sections from each mouse were imaged using a Nikon Eclipse 80i microscope. The adventitial layer was defined by encircling a line around the borders of the outer layer of the aortic section. The medial layer was defined by encircling a line around the borders of the middle layer of the aortic section. Analysis was performed using Nikon Elements Version 3.2.

RNA extraction and DNA microarrays. XY and XX male mice (8 weeks of age; n = 4-5 mice/ genotype) underwent sham surgery or ORC, and after two weeks, mice were placed on Western diet (TD88137, Harlan Teklad, Indianapolis, IN) for one week. RNA from abdominal aortas was extracted using the RNeasy fibrous tissue mini kit (Qiagen, cat # 74704). Harvested abdominal aortic RNA quality and quantity were measured by Agilent 2100 Bioanalyzer using RNA 6000 Nano Labchip kits (Agilent Technologies, Cat # 5067-151). One RNA sample (subject #9) was excluded due to poor quality. The remaining 17 samples had excellent quality and did not differ significantly by treatment group (RNA Integrity Number [RIN]: 9.64 ± 0.03; RNA concentration (ng/ul): 55.7 ± 7.4; 28s:18s ratio: 3.00 ± 0.07; all p-values for main effects of gene and surgery, as well as of interaction terms were n.s. [$p > 0.1$]). Extracted RNA was labeled and hybridized to Affymetrix Mouse Transcriptome Assay 1.0 arrays (one array per subject; n = 17). Signal intensities were calculated using the RMA algorithm[3] at the transcript level using Genomics Suite software (Partek, St Louis). Data were transferred to flat files in Excel and associated with vendor-provided annotation data. Pre-statistical filtering retained unique, annotated probe sets with adequate signal intensity (signal intensity ≥ 4.2 on at least 3 arrays in the study). Filtered signal intensities were analyzed by two-way ANOVA to identify significant main effects of genotype (XY *versus* XX), surgery (Sham vs ORC), as well as interaction. The False Discovery Rate (FDR) procedure[4] was used to estimate the error of multiple testing. The complete list of significant results is provided as supplemental information (Supplemental Table I). Functional categorization for each expression pattern was determined with the prestatistically filtered gene list as a background using DAVID bioinformatic tools [5]. Currently, DAVID does not support Affymetrix MTA 1.0 IDs, and therefore best match IDs from Affymetrix Mouse 1.0 Exon arrays were used, covering more than 90% of the filtered MTA data set. Raw data are available through the Gene Expression Omnibus (GSE #:81580 www.ncbi.nlm.nih.gov/geo/).

Real Time RT-PCR. After 1-day of saline or AngII infusion to XY and XX male *Ldlr*$^{-/-}$ mice (n = 4-6 mice/ genotype/ treatment) RNA was extracted from abdominal and thoracic regions of the aorta using the RNeasy fibrous tissue mini kit (Qiagen, cat # 74704). cDNA was then synthesized using the qScript cDNA Supermix (Quanta Biosciences, cat# 95048-500). Real-time PCR was processed using the SYBER Green FastMix (Quanta Biosciences, cat# 95071-

012) on a BioRad quantitative real-time PCR. mRNA abundance was determined using the ΔΔCt method. Gene expression was normalized to the housekeeping gene, beta-2-microglobulin. Primers sequences for the probed genes are listed in Supplemental Table II.

Statistical Analyses. Data are illustrated as mean ± SEM. Data were tested statistically for normality. If data were not normally distributed, data were statistically transformed and re-analyzed for normality. Data were analyzed using unpaired Student t-tests for two groups, by two-way repeated measures ANOVA on time for longitudinal data with between group factors of surgery or genotype, or by two or three-way ANOVA with between group factors of surgery, region, treatment or genotype. If differences existed between groups, a Holm-Sidak post hoc analysis was performed. Incidence of AAAs and TAAs were analyzed by Fisher's exact test when two groups were examined. Statistical analysis was performed using SigmaPlot software (Version 12.3) or GraphPad Prism 5. $P \leq 0.05$ was considered significant.

References

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