SUPPLEMENTAL MATERIAL

ACE2 Decreases the Formation and Severity of Angiotensin II-induced Abdominal Aortic Aneurysms

Sean E. Thatcher[1], Xuan Zhang[2], Deborah A. Howatt[3], Frederique Yiannikouris[1], Susan B. Gurley[4], Terri Ennis[5], John A. Curci[5], Alan Daugherty[3], and Lisa A. Cassis[1]

Supplemental Materials and Methods

Experimental Animal Models. Male *Ace2*$^{+/y}$ (ACE2 is an X-linked gene) or *Ace2*$^{-/y}$ on an *Ldlr*$^{-/-}$ background (10 times backcrossed on a C57BL/6 background; 8-12 weeks of age) were bred in-house and littermate controls were used for all studies. For each study, mice were fed a high-fat diet (42% kcal from fat, Teklad Diets, TD.88137) beginning 1 week prior to minipump implantations until study endpoint. AngII (1,000 ng/kg/min; Bachem) was infused (Alzet, Model 1004, Durect Corporation) into mice for 28 days. Bone marrow transplantation was performed as described previously,[1] and as described in detail in Supplemental Materials. Vehicle (antipyrin, Sigma, A5882, drug stabilizer in 0.9% saline) or DIZE (30 mg/kg/day, Sigma, D7770):antipyrin (1:1 ratio) were administered daily by intramuscular injection to *Ace2*$^{+y}$ and $^{-/y}$ mice for 7 days prior to implantation of minipumps containing AngII (1,000 ng/kg/min) and throughout the 28-day AngII-infusion protocol. All experiments involving mice conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Elastase AAA model. *Ace2*$^{+/y}$ and $^{-/y}$ *Ldlr*$^{-/-}$ male mice, aged 6-12 months, are anesthetized with 2% isoflurane. After determination of surgical anesthesia, the abdomen and inguinal area are clipped with a #40 blade and then the mouse is placed on its back and secured to the operating tray. The mouse is then scrubbed and prepped with Betadine and alcohol. A midline abdominal incision is made using sterile instruments. The wound is retracted with two clips to expose the abdominal cavity. The mouse is draped with sterile gauze. The intestines will be retracted and placed to the mouse's left, placed in sterile gauze and kept moist. The infrarenal aorta will be exposed from the left renal vein to the aortic bifurcation. Posterior lumbar artery branches and the inferior mesenteric artery will be ligated with 9-0 suture. The aorta will be circumferentially dissected at the proximal and distal ends to allow placement of 6-0 silk sutures to be used as clamps. The aortic diameter will be measured using the micrometer within the microscope eyepiece. The proximal clamp then will be tightened, a microclip placed at the iliac junction, and a small aortotomy will be made using the tip of a 30g needle. The tip of a custom made microrenathane catheter .010 diameter (Braintree) will be placed into the aortotomy and the distal silk tie will be tightened to hold the intraluminal catheter in place. The aorta will be perfused for 5 ½ minutes with 0.49 ml of elastase solution via infusion pump. Following the 5 ½ minute perfusion, the distal tie will be released, the catheter tubing removed, and the aortotomy closed with 10-0 suture. Then the distal clamp will be removed, followed by removal of the proximal tie. After assessment of a patent, non-leaking aorta, and collecting post perfusion measurements of the aorta, the intestines will be replaced and the wound closed in two layers with 4-0 Vicryl and 4-0 nylon suture. Following surgery, prior to closure, the mouse may be given sterile warmed fluids, 0.25-1 ml, IP to replace minimal blood loss and to remoisten the abdominal cavity. The mouse will be placed on a circulating heated water blanket and allowed to recover. As described above, aseptic technique and postoperative care will be given.

Measurements of Vascular Pathologies. Ultrasound measurements (Vevo 660 or 2100) were performed at baseline (day 0 of infusion), and during AngII infusions on day 14 and 27. Mice exhibiting a 50% increase in aortic lumen diameter compared to baseline were defined as exhibiting an AAA. At the end of the study, mice were euthanized with a lethal dose of ketamine/xylazine mix (100/10 mg/kg, ip) and tissues (spleen, kidney, liver) were snap-frozen in liquid nitrogen. Aortas were placed in 10% formalin and cleaned of adherent tissue to quantify maximal external diameters of suprarenal aortas of mice exhibiting an AAA as described previously.[2]

Measurements of Systolic Blood Pressure. Systolic blood pressure was quantified by tail cuff as described previously.[1] Mice were acclimated to the system prior to and during week 3 of AngII-infusion and recordings were obtained for 5 consecutive days.

Genotyping by polymerase chain reaction. C57BL/6J mice (ten times backcrossed) were provided by Drs. Thomas Coffman and Susan Gurley from Duke University.[3] *Ace2*$^{+/y}$ or $^{-/y}$ males were crossed to *Ldlr*$^{-/-}$ female mice until breeding pairs were established. Tail DNA was obtained by using the Qiagen DNeasy Tissue and Blood kit (Qiagen, cat#69506). Mice were screened for *Ace2* and *Ldlr* deficiency using primers listed, (*Ace2*$^{+/y}$, Forward primer, 5'-GGGCCAGAGTATCTGCCCAG-3' Reverse primer, 5'-GCAGGATCTCCTGTCATCTCACC-3' 380 bp)(*Ace2*$^{-/y}$, Forward primer, 5'-TCGCCTTCTATCGCCTTCTTGAC-3' Reverse primer, 5'-TTGGCGGATAATGCCTTTAG-3' 583 bp)(*Ldlr*, neo cassette primer, 5'-AATCCATCTTGTTCAATGGCCGATC-3', upstream of neo cassette, 5'-CCATATGCATCCCCAGTCTT-3', exon 4, 5'-GCGATGGATACACTCACTGC-3', *Ldlr*$^{+/+}$ 167 bp; *Ldlr*$^{-/-}$ 350 bp).

Bone marrow transplantation. Male *Ldlr*$^{-/-}$ mice (8 weeks old) were irradiated lethally with a total of 900 rads divided into two doses (450 rads/dose; 3 hours apart) from a cesium γ source. Bone marrow-derived cells were harvested from femurs of *Ace2*$^{+/y}$ or $^{-/y}$ mice on an *Ldlr*$^{-/-}$ background and injected into irradiated recipient *Ldlr*$^{-/-}$ mice (1×10^7 donor cells/mouse). Eight weeks after irradiation, recipient mice were fed a high fat diet (42% caloric intake from fat, TD88137, Harlan Teklad, Indianapolis, IN) beginning 1 week prior to infusions of AngII (1,000 ng/kg/min). After completion of AngII infusions, DNA was isolated from bone marrows of anesthetized (ketamine/xylazine, 100/10 mg/kg, ip) recipient mice and PCR was performed to verify the successful repopulation of donor cells.

ACE2 mRNA and activity measurements. Mouse kidneys were dissected in half and one portion was used for total RNA extraction and the other for ACE2 activity. RNA extraction was performed using a kit (Promega, Total RNA extraction, cat#Z3105). RNA was quantified by spectrophotometry using a Nanodrop 2000 (ThermoScientific), diluted to 0.4 µg per reaction and reverse transcribed using a cDNA synthesis kit (qScript cDNA Supermix, Quanta Biosciences, cat#95048-500). cDNA was diluted at a ratio of 1:50 (0.4 ng/µL) and 5 µLs were used per PCR reaction (PerfeCta SYBR Green FastMix for iQ, Quanta Biosciences, cat#95071-012). ACE2 and 18S mRNA abundances were analyzed using the $2^{-\Delta\Delta Ct}$ method. Primers sequences were

described previously.[4] ACE2 enzymatic activity was quantified as described previously.[5] Briefly, samples were placed in a 0.5 mL Tris-NaCl solution containing 10 μM of $ZnCl_2$ and Z-pro-prolinal (ACE2 buffer) and homogenized with metal beads using a GenoGrinder for 30 seconds at 1,250 RPMs. Samples were centrifuged at 14,000 RPMs for 20 minutes at 4°C. Supernatants were discarded and the pellets were diluted with 0.5 mL of 0.5% Triton-X in ACE2 buffer. Samples were vortexed and refrigerated (4°C) overnight. The next day, samples were centrifuged at 5,000 RPMs for 10 minutes (4°C). The supernatant was used for quantifying ACE2 activity and protein was determined using a BCA kit (Thermoscientific, cat#23225) with BSA used as the standard (BioRad, cat#500-0007). We quantified ACE2 activity using 0.01 mg of kidney protein in buffer (total volume of 250 μL; Tris-NaCl buffer containing 1 μM pepstatin A, 10 μM captopril, 100 μM bestatin hydrochloride, 10 μM phospharamidon, and 10 μM thiorphan). Samples were incubated with ^{125}I-AngII (specific radioactivity, 2,200 Ci/mmol) for 30 minutes at 37°C. Reactions were stopped with 50 μL of 1% phosphoric acid and frozen at -20°C until angiotensin peptides could be resolved by HPLC (AngII, Ang-(1-7) were resolved using HPLC as described previously).[5] HPLC fractions (1 ml) were collected and analyzed by gamma spectrometry to quantify radioactivity within fractions containing AngII or Ang-(1-7). ACE2 activity is expressed as femtomoles per milligram protein per minute, based on the specific activity of [^{125}I]AngII (2,175 Ci/mmol).

Plasma and Serum Component Analysis. Plasma renin concentrations were quantified as described previously.[1] Plasma concentrations of Ang-(1-7) were quantified using a commercial ELISA kit (Bachem, cat#S-1330) as described previously.[6] Sera cholesterol and triglyceride concentrations were quantified using a commercial kit (Total Cholesterol E kit, Wako cat#439-17501; Total Serum Triglycerides kit, Wako cat#290-63701) as described previously.[7] To quantify lipoprotein cholesterol concentrations, fast protein liquid chromatography (FPLC) was performed on individual serum samples (n = 4 mice/group) to resolve lipoproteins, and cholesterol was quantified in individual fractions as described previously.[8] Measurements were imported into PeakFit (v.4.12) and chylomicrons (CM)/VLDL, LDL, and HDL cholesterol concentrations were determined from areas under the curve (AUC). AUCs were calculated and multiplied to total serum cholesterol concentrations to determine cholesterol content (mg/dl) for each lipoprotein fraction.[8]

Immunohistochemistry of murine AAAs. A 3% low, melting-point agarose solution was made and a green marking tissue dye was added (Polysciences Inc., cat#24110). Mice were perfused with a 10% formalin solution for approximately 15 minutes at physiological pressure and peripheral organs were removed. A 1 mL syringe with needle (gauge 23) was filled with green agarose solution and perfused slowly via the left ventricle. Aortas were cleaned of adherent tissue. Abdominal aorta was placed in 30% sucrose solution until the tissue sank to the bottom of a 15 mL conical tube. Abdominal segments were then placed in OCT media and serially sectioned (10 μm). Sections were placed on positively charged slides (Probe On Plus, Fischer Scientific, cat#22-230-900) and stained using the Microprobe system (Fischer Scientific). Sections were stained with Gomori's Trichrome stain as described previously.[9] For ACE2 immunostaining, sections were cleared using 100% xylene before using a step-down

series of alcohol-water mixes (100%, 95%, 75%, 100% water). Sections were then incubated with 0.05% chromic acid for 2 minutes at 40°C for 2 minutes followed by a rinse in 1X automation buffer (10X automation buffer, GeneTex, cat#GTX30931). Antigen retrieval was then performed using a 1:100 dilution of warmed citrate buffer (low pH citrate buffer, Vector Labs, cat#H-3300) for 10 minutes followed by a 10 minute incubation at room temperature. Sections were then washed 4 times in automation buffer and then pre-incubated with 1% hydrogen peroxide in methanol for 3 minutes at 40°C. Sections were again washed with automation buffer and blocked with goat serum for 5 minutes at 40°C. Sections were then incubated with a monoclonal goat, anti-rabbit IgG for ACE2 for 30 minutes at 40°C (anti-rabbit, Abcam, cat#ab15348, 1mg/mL, stock)(final concentration 20 µg). A negative IgG control was used through a non-immune rabbit IgG at the same concentration as the ACE2 antibody (GeneTex, cat#GTX35035). Sections were rinsed with automation buffer and incubated with a biotinlayted goat anti-rabbit IgG linked to horseradish peroxidase (HRP)(Vector Labs, cat#BA-1000, final concentration 7.5 µg/mL) for 30 minutes at 40°C. Sections were rinsed again and an ABC kit was utilized for avidin-biotin conjugation for 30 minutes at room temperature (Vector Lab, cat#PK-6100). Sections were rinsed and a peroxidase enhancer step was used as a subsequent wash (GeneTex, cat#GTX82979). Finally, an AEC kit (Vector Labs, cat#SK-4200) was used for detection of antibody-peroxidase complex and counterstained with hematoxylin. Glycerol-gelatin (Sigma, cat#GG-15ML) was heated and used as a mounting medium for slides. For macrophage staining, CD68 was utilized (anti-rat, Abcam, clone FA-11, cat#ab53444, 1 mg/mL, stock)(final concentration 20 µg). Staining was performed in the same manner as ACE2, however the antigen retrieval step and counter staining with hematoxylin was omitted. For macrophage quantification, a similar protocol was used for detection of macrophages at the medial break.[10] All images were taken at the same exposure settings and images were threshold to include positive staining (red-brown) and areas were summed to get the total macrophage area in mm^2.

Immunohistochemistry of human AAAs. The same protocol used in ACE2 immunostaining of murine AAAs (see above) was utilized in staining human abdominal aortic sections. CD68 immunostaining was performed using a monoclonal mouse anti-human antibody (clone KP1, DakoCytomation, cat#M 0814) using a peroxidase-conjugated ImmPRESS anti-mouse IgG (Vector Labs, cat#MP-7402). The reactions were visualized using ImmPACT DAB peroxidase substrate (Vector Labs, cat#SK-4105). Negative control for CD68 was incubation with the secondary only. Human AAA sections were surgical samples procured at Washington University, MO.

Sample	Age	Gender
Non-AAA #1	56	M
Non-AAA #2	64	F
Non-AAA #3	46	M
Non-AAA #4	51	M
Non-AAA #5	52	F
Non-AAA #6	53	M
AAA #1	70	F
AAA #2	61	M

AAA #3	66	F
AAA #4	72	F
AAA #5	79	M
AAA #6	68	M

Statistics. Data are represented as mean ± SEM. For data that passed normality and equal variance, a Student's t-test was used to evaluate differences between genotypes or in mice administered vehicle versus DIZE. For two group comparisons where data did not pass normality and/or equal variances, a Mann-Whitney U test was performed. Ultrasound measurements were analyzed by repeated measures two-way ANOVA followed by a pairwise multiple comparison test (Holm-Sidak). A Fischer's exact test was used to analyze AAA incidence between groups of mice. Statistical analysis was performed through SigmaPlot (v.12) with significance at $P < 0.05$.

References

1. Cassis LA, Rateri DL, Lu H, Daugherty A. Bone marrow transplantation reveals that recipient at1a receptors are required to initiate angiotensin ii-induced atherosclerosis and aneurysms. *Arterioscler Thromb Vasc Biol*. 2007;27:380-386
2. Police SB, Thatcher SE, Charnigo R, Daugherty A, Cassis LA. Obesity promotes inflammation in periaortic adipose tissue and angiotensin ii-induced abdominal aortic aneurysm formation. *Arterioscler Thromb Vasc Biol*. 2009;29:1458-1464
3. Gurley SB, Allred A, Le TH, Griffiths R, Mao L, Philip N, Haystead TA, Donoghue M, Breitbart RE, Acton SL, Rockman HA, Coffman TM. Altered blood pressure responses and normal cardiac phenotype in ace2-null mice. *J Clin Invest*. 2006;116:2218-2225
4. Thatcher SE, Gupte M, Hatch N, Cassis LA. Deficiency of ace2 in bone-marrow-derived cells increases expression of tnf-alpha in adipose stromal cells and augments glucose intolerance in obese c57bl/6 mice. *Int J Hypertens*. 2012;2012:762094
5. Gupte M, Boustany-Kari CM, Bharadwaj K, Police S, Thatcher S, Gong MC, English VL, Cassis LA. Ace2 is expressed in mouse adipocytes and regulated by a high-fat diet. *Am J Physiol Regul Integr Comp Physiol*. 2008;295:R781-788
6. Thatcher SE, Zhang X, Howatt DA, Lu H, Gurley SB, Daugherty A, Cassis LA. Angiotensin-converting enzyme 2 deficiency in whole body or bone marrow-derived cells increases atherosclerosis in low-density lipoprotein receptor-/- mice. *Arterioscler Thromb Vasc Biol*. 2011;31:758-765
7. Daugherty A, Rateri DL, Lu H, Inagami T, Cassis LA. Hypercholesterolemia stimulates angiotensin peptide synthesis and contributes to atherosclerosis through the at1a receptor. *Circulation*. 2004;110:3849-3857
8. Wang S, Subramanian V, Lu H, Howatt DA, Moorleghen JJ, Charnigo R, Cassis LA, Daugherty A. Deficiency of receptor-associated protein attenuates angiotensin ii-induced atherosclerosis in hypercholesterolemic mice without influencing abdominal aortic aneurysms. *Atherosclerosis*. 2012;220:375-380
9. Gomori G. A rapid one-step trichrome stain. *Am J Clin Pathol*. 1950;20:661-664
10. Zhang X, Thatcher S, Wu C, Daugherty A, Cassis LA. Castration of male mice prevents the progression of established angiotensin ii-induced abdominal aortic aneurysms. *J Vasc Surg*. 2014