Materials and Methods

Animals

Mice deficient in both acute phase SAA isoforms, SAA1.1 and SAA2.1[1] were bred to obtain a > 99.9% C57BL/6 background using the Jackson Laboratory Speed Congenic Service to expedite the process. The mice were then crossed with apoE$^{-/-}$ mice (C57BL/6 background, originally obtained from the Jackson Laboratory; Bar Harbor, Maine) to generate apoE$^{-/-}$ mice lacking acute phase SAAs. For simplicity, apoE$^{-/-}$ mice and apoE$^{-/-}$ mice lacking SAA1.1/SAA2.1 are referred to as SAAWT and SAAKO mice, respectively. The genotype of all mice was verified by PCR as previously described[1]. To confirm the absence of both SAA1.1 and SAA2.1 proteins in SAAKO mice, plasma was collected from randomly selected mice 24 hr after LPS injection and analyzed by isoelectric focusing and Western blotting (data not shown). Animals were housed in microisolator cages and provided with normal rodent diet and water *ad libitum*. AngII (1,000 ng kg^{-1} min^{-1}; Sigma-Aldrich, St. Louis, MO) or saline was administered via Alzet osmotic minipumps (model 2004; Durect Corporation, Cupertino, CA) to 12-14 week old male mice anesthetized with 50 µl ketamine/Xylazine, 90/10 mg/ml respectively (Ketamine, Butler Schein, Dublin, OH; Xylazine, Lloyd laboratories, Shenandoah, IA) as previously described[2]. All mice that died during the course of AngII infusion underwent necropsy to determine the cause of death. All studies were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee.

Blood pressure measurements

Systolic blood pressure was measured on 5 consecutive days in conscious mice using a non-invasive tail-cuff method (CODA 8; Kent Scientific Corp, Torrington, CT) as described[3]. Baseline measurements were obtained the week prior to osmotic minipump implantation, and endpoint measurements during the last week of AngII infusion.

Quantification of AAAs and atherosclerotic lesions

Abdominal aortas were visualized in mice anesthetized with Isofluorane (Butler Schein, Dublin,OH) using high frequency ultrasound (US) (Vevo 660; VisualSonics, Toronto, ON, Canada) as described previously[4]. Maximal luminal diameter and area measurements were derived from suprarenal aortic images obtained the day of osmotic minipump implantation and after 9 or 27 days of AngII infusion. AAAs were also quantified *ex vivo*

by measuring maximal diameters of suprarenal aortas. For quantification of atherosclerosis, aortas were cleaned of adventitial tissue, cut open longitudinally and pinned *en face* to expose intimal surfaces. Aneurysms in the abdominal aorta permitted atherosclerotic lesion analyses only in the aortic arch, defined as the region from the ascending aorta to 3mm distal to the subclavian artery. Lesion area and aortic arch luminal surface area were quantified using ImagePro Plus software (Media Cybernetics, Bethesda, MD) as previously described[5].

Plasma SAA, IL-6 and renin measurements

Plasma SAA concentrations were determined using a mouse SAA ELISA kit (cat no TP 802M, Tridelta Development Ltd). Plasma IL-6 was measured using a mouse IL-6 ELISA kit (cat no M60008, R&D Systems). Plasma renin activity was measured as previously described[6]. Briefly, mouse plasma (8 µl) was incubated for 30 min at 37°C with 25 nM exogenous angiotensinogen, prepared from nephrectomized rats. Angiotensin I, produced from angiotensinogen, is a measure of renin present in the sample and was quantified by radioimmunoassay (GammaCoat® Plasma Renin Activity Radioimmunoassay Kit CA1553, DiaSorin Inc. MN).

Immunohistochemistry (IHC)

Human pathological tissue was obtained from archived samples that were collected following a protocol approved by the Washington University School of Medicine Human Research Subjects Committee. Formalin-fixed human aortic tissues were embedded in paraffin for sectioning. Abdominal aortae from AngII-infused SAAWT mice were frozen in Optimal Cutting Temperature compound (OCT; Tissue-Tek, Torrance, CA) and 8 µm thick sections down the length of the aorta were mounted on glass slides. Sections were fixed in 4% paraformaldehyde and incubated with 3% H_2O_2 for 10 min to quench endogenous peroxidases. After blocking endogenous biotin (Avidin/Biotin blocking kit, Vector Laboratories, Burlingame, CA), slides were incubated overnight at 4°C with rabbit anti-mouse SAA (1:500 dilution, de Beer laboratory) or rat anti-mouse CD68 (1:200, ab53444, Abcam, Cambridge, MA). Human sections were incubated with rabbit anti-human SAA (1:1,000 dilution, de Beer laboratory or control antiserum 1:1,000 dilution). Secondary biotinylated antibody and avidin-peroxidase were applied according to vendor's instructions (Vectastain Elite ABC kit, Vector Laboratories Inc., Burlingame, CA). Immunoreactivity was visualized using an AEC kit (SK-4200, Vector Laboratories

Inc., Burlingame, CA), followed by counterstaining with hematoxylin. Immunoreactivity was visualized using the red chromogen, 3-amino-9-ethyl carbazole (Vector Laboratories Inc., Burlingame, CA). For elastin staining, OCT-embedded sections were fixed in 10% formalin and treated according to instructions (Elastic Stain Kit, Thermo Fisher Scientific, Kalamazoo, MI). Images were captured on a Nikon ECLIPSE 80i microscope with the aid of NIS-Elements BR 4.00.08 software.

Zymography

Gel zymography was performed as described previously[5]. Aortas were rapidly cleaned of adventitial tissue and separated into thoracic aorta, defined as the region from the aortic root to the diaphragm, and the abdominal aorta, defined as the region from the diaphragm to the ileal bifurcation. Aortas were homogenized in 0.08 mL lysis buffer (Cell Signaling, cat no 9803) containing a protease inhibitor cocktail (Sigma cat no P8340). Protein (5 – 15 µg) was electrophoresed on a 7.5% SDS-polyacrylamide gel containing 2 mg/ml gelatin. Gels were renatured in 2.5% Triton X-100 for 1 h, washed twice for 15 min with dH_2O, and then incubated in 50 mmol/L Tris–HCl containing 5 mmol/L $CaCl_2$ for 24 h prior to staining with Coomassie Brilliant Blue. The relative densities of the 70kDa, 64kDa and 58kDa forms of MMP-2 in aortas of SAAWT mice were arbitrarily given a value of 1, and the amount of 70kDa, 64kDa and 58kDa MMP2 in SAAKO mice were expressed relative to that of SAAWT mice.

For *in situ* zymography, OCT-embedded abdominal aorta sections, adjacent to those used for IHC (see above), were incubated with 20 µg/ml DQ gelatin fluorescein conjugate for 2 h at 37°C according to kit instructions (EnzChek Gelatinase/Collagenase Assay Kit, cat no E-12055, Molecular Probes, Inc. Eugene, OR). The fluorescence generated by hydrolysis of the added substrate was recorded by an Olympus IX70 microscope equipped with Olympus DP70 digital camera. The general MMP inhibitor 1,10-phenanthroline, 20 mmol/L, was used to define non-specific fluorescence (Sigma-Aldrich, St Louis, MO).

SAA purification

Mouse SAA was purified as described[7] from HDL isolated from mouse plasma collected 24 h after injection with 1-2.5 µg/kg *Escherichia coli* lipopolysaccharide (LPS; 0111:B4, Sigma-Aldrich , St Louis MO) or 0.5 ml 2% $AgNO_3$ (cat S1115, Spectrum). Briefly, ~20

mg HDL was delipidated with EtOH:ether (3:2) and the delipidated proteins were separated by gel filtration on a Sephacryl S-200 column in buffer containing 7 mol/L urea, 20 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L EDTA, pH 8.4. SAA-containing fractions were identified by SDS PAGE, pooled and dialyzed against 2 mmol/L Tris, 15 mmol/L NaCl, and 0.1 mmol/L EDTA pH 8.4 prior to 10-fold concentration. LPS contamination in purified SAA preparations was below the level of detection (ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit, cat no L00350C, GenScript, Piscataway,NJ).

Cell Culture

J774 cells: J774 macrophage-like cells were obtained from the American Type Culture Collection and maintained in DMEM (Dulbecco's Modified Eagle's Medium containing 100 U/ml penicillin, 100 μg/ml streptomycin) supplemented with 10% fetal bovine serum (FBS) and 2 mmol/ L-glutamine. For SAA incubations, J774 cells were grown to 90% confluence in 12-well clusters.

Mouse aortic vascular smooth muscle cells (VSMC): Aortic VSMC were isolated from C57BL/6 mice as described[8]. Mice were euthanized with 300 μl ketamine/Xylazine, 90/10 mg/ml, respectively. Aortas were rapidly excised and the adventitia removed with the aid of a dissecting microscope. Dissected aortas were washed briefly in serum-free DMEM. The vessels were incised longitudinally, further cleaned and then dissociated at 37°C with 1 mg/ml collagenase type I (Worthington Biochemical Corp, Lakewood, NJ) and 0.125 mg/ml elastase type III (Sigma-Aldrich). After 40 min incubation, the digests were pipetted vigorously every 10 min for another 40 min. Cell suspensions were centrifuged at 3000 rpm for 10 min at 4°C and cell pellets resuspended in DMEM containing 20% FBS. Purified cells were thoroughly dispersed by repeated gentle pipetting then plated and cultured in 6-well clusters for 3 days prior to use. One aorta provided sufficient cells for one well of a 6-well cluster.

For SAA treatments, VSMC or J774 cells were washed 3 times with serum-free DMEM prior to incubating for 8 h with the same media (0.8 ml and 1.6 ml for 12-well and 6-well clusters, respectively) containing 50 μg/ml purified mouse SAA. Cells were subsequently washed once with phosphate-buffered saline and solubilized in 350 μl RLT buffer for RNA isolation according to manufacturer's instructions (RNeasy® Mini Kit, cat no 74106, Qiagen)

RNA isolation and quantitative RT-PCR

Total RNA was isolated from mouse livers and cultured cells according to the manufacturer's instructions (RNeasy® Mini Kit, cat no 74106, Qiagen). RNA samples were incubated with DNase I (cat no 79254, Qiagen) for 15 min at RT prior to reverse transcription. Liver RNA (2 µg) or RNA from cultured cells (0.5µg) was reverse transcribed into cDNA using the Reverse Transcription System (cat no 4368814, Applied Biosystems). After 4-fold dilution, 5 ul was used as a template for real-time RT-PCR. Amplification was done for 40 cycles using Power SYBR Green PCR master Mix Kit (cat no 4367659, Applied Biosystems). Quantification of mRNA was performed using the $\Delta\Delta C_T$ method and normalized to GAPDH. Primer sequences are provided in Supplemental Table I.

Statistical analyses

Numeric data are summarized as mean ± SEM. Comparisons of two groups defined by genotype or treatment were performed using unpaired student's t test (after logarithmic transformation for figures 2C and 2D) or the nonparametric rank sum test for numeric data and using Fisher's exact test for aneurysm incidence data. Comparisons of four groups defined by genotype and treatment were performed using two-way ANOVA, with observation weights based on within-group variances; in the event of a significant interaction, comparisons of groups with a common treatment and of groups with a common genotype were performed using linear contrasts. Blood pressure data were analyzed using a linear mixed model with time and genotype as predictor variables and with random effects for individual animals. Values of $P<0.05$ were considered statistically significant.

References

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