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Inducible Depletion of Calpain-2 Mitigates Abdominal Aortic Aneurysm in Mice

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Abstract

Objective: Cytoskeletal structural proteins maintain cell structural integrity by bridging extracellular matrix (ECM) with contractile filaments. During AAA development, (i) aortic medial degeneration is associated with loss of smooth muscle cell (SMC) integrity, and (ii) fibrogenic mesenchymal cells (FMSCs) mediate ECM remodeling. Calpains cleave cytoskeletal proteins that maintain cell structural integrity. Pharmacological inhibition of calpains exert beneficial effects on Angiotensin II (AngII)-induced AAAs in low density receptor deficient (LDLR−/−) mice. Here we evaluated the functional contribution of FMSCs-derived calpain-2 on (i) cytoskeletal structural protein and ECM alterations, and (ii) AAA progression.

Approach and Results: Calpain-2 protein, and cytoskeletal protein (filamin and talin) fragmentation are significantly elevated in human and AngII-induced AAAs in mice. To examine the relative contribution of calpain-2 in AAA development, calpain-2 floxed mice in an LDLr−/− background were bred to mice with a tamoxifen-inducible form of Cre under control of either the ubiquitous promoter, chicken β-actin or FMSC-specific promoter, Col1α2. Ubiquitous or FMSC-specific depletion of calpain-2 in mice suppressed AngII-induced AAAs, filamin/talin fragmentation, while promoting ECM protein, collagen in the aortas. Calpain-2 silencing in aortic SMCs or fibroblasts reduced AngII-induced filamin fragmentation. In addition, silencing of filamin in aortic SMCs significantly reduced collagen protein. Furthermore, calpain-2 deficiency suppressed rupture of established AngII-induced AAAs in mice.

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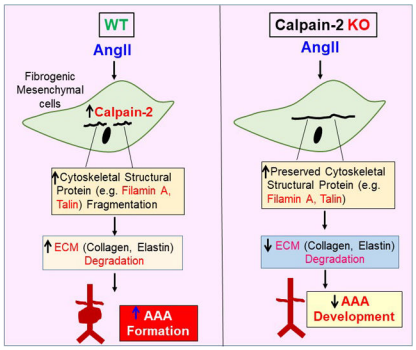
Conflicts of Interest: None

DISCLOSURES
None.

Conclusion: Our studies implicate that calpain-2 deficiency prevents (i) AngII-induced cytoskeletal structural protein fragmentation and AAA development, and (ii) stabilize and suppress rupture of established AAAs in mice.

Graphical Abstract



Keywords

Angiotensin II; calpain; aneurysm; cytoskeletal protein

Subject codes:

Aneurysm

INTRODUCTION

Abdominal aortic aneurysms (AAAs) are permanent dilations of the abdominal aorta with greater than 80% mortality after rupture.[1] Lack of sufficient mechanistic knowledge and availability of specific therapeutic agents to stabilize and /or prevent aortic rupture, highlight the need to gain mechanistic insights into AAA development to improve the long-term prognosis. Loss of smooth muscle cell (SMC) integrity is associated with aortic medial degeneration during AAA formation.[2, 3] Cytoskeletal structural proteins maintain cell structural integrity by bridging the extracellular matrix (ECM) with SMC contractile filaments.[4, 5] Activated mesenchymal cells which includes adventitial fibroblasts, myofibroblasts and a subpopulation of synthetic medial SMCs play a potent role on ECM remodeling. These heterogenous lineage cells secrete ECM protein, type I collagen which is composed of α1 and α2 peptides, and are encoded by Col1α1 and Col1α2.[6] Currently, it is not clear whether cytoskeletal structural proteins play a role in vessel wall integrity during AAA development, and the identity of intracellular proteases especially of fibrogenic mesenchymal cells origin that target cytoskeletal structural proteins remain undefined.

Calpains, calcium-dependent cysteine proteases, tightly regulate target protein substrates through limited proteolysis.[7] The two major isoforms, calpain-1 and −2, are expressed ubiquitously; whereas the other isoforms (e.g. −3, −9) are tissue-specific.[7] Calpains are the only known intracellular proteases that target an array of cytoskeletal and membrane proteins.[8, 9] Earlier, we demonstrated that pharmacological inhibition of calpain-1 and −2

reduced angiotensin II (AngII)-induced AAAs in mice.[10] In addition, calpain-2 compensated for loss of calpain-1 and promoted AAA formation in calpain-1 deficient mice.[11] Furthermore, using leukocyte-specific calpain-2 deficient mice, we demonstrated that leukocyte-derived calpain-2 had no influence on AngII-induced AAA development in mice.[12]

In this study, using human AAA tissue samples, we examined calpain-2 protein and activity and fragmentation of cytoskeletal structural proteins, filamin A and talin. In addition, to elucidate the functional role of calpain-2 in AngII-induced AAA development and cytoskeletal protein fragmentation, we generated an inducible whole body or mesenchymal-specific calpain-2 deficient mice in an LDL receptor deficient background. These studies demonstrated that (i) calpain-2 protein and activity, and cytoskeletal structural protein fragmentation were increased in human and experimental AAAs, (ii) induced depletion of calpain-2 strongly suppressed AAA development and cytoskeletal protein fragmentation in mice, (iii) mesenchymal derived calpain-2 deficiency strongly suppressed AAA development in mice. Furthermore, siRNA mediated silencing of calpain-2 in aortic SMCs and fibroblasts prevented AngII-induced filamin fragmentation. In addition, to elucidate the functional role of cytoskeletal structural protein, filamin A, on ECM protein stability, we examined the effect of filamin A silencing in aortic SMCs. These studies demonstrated that silencing of filamin A significantly reduced abundance of collagen protein in aortic SMCs. Furthermore, inducible depletion of calpain-2 also prevented rupture of established AAAs with improved survival rate.

METHODS

The data, analytic methods, and study materials will be maintained by the corresponding author and made available to other researchers upon reasonable request.

Mice

LDL receptor −/− (stock # 002207), inducible β-actin Cre+/0 (CAG-Cre-ER™; stock # 004682), Col1α2 Cre+/0 (Col1a2-Cre-ER, Stock # 029235), ROSA26R^{LacZ} (Stock # 003474) and C57BL/6J (Stock# 000664) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Calpain-2 floxed (f/f) mice on a C57BL/6J background were originally generated in the laboratory of Dr. Takaomi Saido.[13] LDL receptor −/− and calpain-2 f/f mice were backcrossed 10 times into a C57BL/6J background. The β-actin Cre+/0 mice were obtained in the C57BL/6 background and backcrossed 8–10 times into a C57BL/6J background. The Col1α2 Cre+/0 mice were obtained in the C57BL/6 × DBA/2 background and backcrossed 8 times into a C57BL/6J background.

Both calpain-2 f/f and β-actin/ Col1α2 Cre+/0 mice were bred to an LDL receptor −/− background. Female calpain-2 f/f mice were bred with male Cre+/0 mice to yield mice homologous for the floxed calpain-2 gene and hemizygous for the Cre transgene. Littermates that were homozygous for the floxed calpain-2 gene, but without the Cre transgene (Cre0/0), were used as control mice.

Since AAAs exhibit sexual dimorphism, with predominance occurring among men, only age-matched male littermates (8–10 weeks old) were used for the present study. Mice were maintained in a barrier facility and fed normal mouse laboratory diet. Ethics and Institutional Review Board approvals were provided by University of Kentucky (Protocol-2011–0907), the Vanderbilt Medical Center (Protocol-141662), and informed consent was received from all participants.

Mouse Genotyping

Mouse genotypes were confirmed by PCR. DNA was isolated from tail snips using a Maxwell tissue DNA kit (Cat# AS1030, Promega, Madison, WI). Calpain-2f/f genotyping used the following primers: 5'-ATAGCTCCTGTGTATCAGGCACAGAGCTGG and 5'-CTCTGGTCAGGTCTTAGTTCCCAGAGGATG. Resultant wild-type, and flox allele bands were 290, and 430 bp, respectively (Figure IA in the Data Supplement). Cre+ genotyping used the following primers: 5'-ACCTGAAGATGTTCGCGATT and 5'-CGGCATCAACGTTTTCTTTT. The resultant Cre+ hemizygous allele PCR product was 182 bp and no product for non-transgenic mice. The IL-2 gene was used as an internal control for Cre+ genotyping using the following primers: 5'-CTAGGCCACAGAATTGAAAGATCT and 5'-GTAGGTGGAAATTCTAGCATCATCC. The resultant product was 324 bp (Figure IB in the Data Supplement). LDL receptor genotyping was performed as described previously.[14]

Tamoxifen Injections

Tamoxifen (Cat# T5648; Sigma Chemical; St. Louis, MO) was dissolved in 100% ethanol (30 mg in 300 μl) and then diluted in corn oil (Cat# C8267; Sigma Chemical; St. Louis, MO) for a final concentration of 10 mg/ml. Mice (6–8 weeks old) were injected i.p. with a dose of tamoxifen of 25 mg/kg daily for 5 consecutive days.

Diet

To induce hypercholesterolemia, mice were fed a diet supplemented with saturated fat (21% wt/wt milk fat; TD.88137, Harlan Teklad, Indianapolis, IN) for 5 or 12 weeks. Diet was started one week before AngII infusion.

AngII Infusion

Two weeks after last tamoxifen injection and an initial week of high-fat diet feeding, mice were implanted with Alzet osmotic minipumps (Model 2004, Durect Corporation, Cupertino, CA), subcutaneously into their right flanks, and infused with either saline or AngII (1,000 ng/kg/min, Bachem, Torrance, CA) continuously for a period of 7, 28 or 84 days, as described previously.[11] Mice were fed a high fat-enriched diet throughout the infusion study.

Blood Pressure Measurement

Systolic blood pressure (SBP) was measured noninvasively on conscious mice by volume pressure recording of the tail using a computerized tail cuff blood pressure system (Kent

Scientific Corp, Torrington, CT).[15] SBP was measured on 3 consecutive days prior to pump implantation, and during the last 3 days of AngII infusion.

Measurement of Plasma Components

Plasma cholesterol concentrations were measured using a commercially available enzymatic kit (Wako, Richmond, VA) as described previously.[14]

Ultrasound Imaging of Abdominal Aortic Aneurysms

Luminal dilation of the abdominal aorta was measured by a high frequency ultrasound imaging system (Vevo 2100, VisualSonics, Toronto, Canada) using a MS400 MicroScanTM transducer with a resolution frequency of 18–38 MHz.[16] Mice were anesthetized and restrained in a supine position to acquire ultrasonic images. Short axis scans of abdominal aortas were performed from the left renal arterial branch level to the suprarenal region.[16] Images of abdominal aortas were acquired and measured to determine maximal diameter in the suprarenal region of the abdominal aorta. Aortic images were acquired at day 0, 28,42 and 70 of AngII-infusion. The AAA incidence is defined as >50% increase in lumen diameter compared with baseline (day 0 of infusion). In addition, AAA rupture is also included in the AAA incidence calculation.

Quantification of Atherosclerosis, Ascending and Abdominal Aortic Aneurysms

After saline was perfused through the left ventricle of the heart, aortas were removed from the origin to iliac bifurcation, and placed in formalin (10% wt/vol) overnight. Adventitial fat was cleaned from the aortas. Atherosclerosis was quantified on aortic arches and thoracic aorta as lesion area, and percent lesion area on the intimal surface by en face analysis as described previously.[17] Lesion areas were measured using Image-Pro Plus software (MediaCybernetics, Bethesda, MD) by direct visualization of lesions under a dissecting microscope. For aneurysm measurements, AAAs were quantified *ex vivo* by measuring the maximum external width of the suprarenal abdominal aortic diameter using computerized morphometry (Image-Pro Cybernetics, Bethesda, MD) as described previously.[11] For ascending AA measurement, intimal areas of ascending aortas were measured from the ascending aorta to the subclavian branch using Image-Pro Plus software.[11, 18]

Tissue Histology

Abdominal aortas were placed in optimal cutting temperature (OCT) compound and serially sectioned (10 μm thickness/section) in sets of 10 slides with 9 sections/slide using a cryostat.[18] One slide of each serial set was stained with Movat's Pentachrome (Polyscientific) to visualize elastin breaks.

β-Galactosidase staining in tissues

Whole tissues were fixed in paraformaldehyde for 1 hour, and preincubated with buffer containing 100 mM sodium phosphate, pH7.3; 2 mM magnesium chloride; 0.01% sodium deoxycholate; 0.02% NP-40 (Cat# S-3139; M-8266; D6750; 74385; Sigma-Aldrich; St. Louis, MO). Subsequently, tissues were incubated overnight in staining buffer (above buffer with additions of 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide; Cat #

702857; P3289; Sigma-Aldrich; St. Louis, MO) containing β-galactosidase (1 mg/ml; Cat # V394A; Promega, Madison, WI). Samples were post fixed in formalin overnight and then stored in ethanol (70%).

Immunohistochemistry of Human AAAs.

Calpain-2 immunostaining was performed using a rabbit anti-human calpain-2 (10 μg/ml, catalog No. RP-3 Calpain-2; Triple Point biologics, Forest Grove, OR). CD68 immunostaining was performed using a rabbit anti-human CD68 antibody (1:200, catalog No. SC-9139; SantaCruz Biotechnology, Dallas, TX). Biotinylated secondary antibodies from the appropriate species were used (Vector Laboratories). A peroxidase-based ABC system (Vector Laboratories) and the red chromogen AEC were used to detect the antigen-antibody reaction. Immunostaining was performed on formalin-fixed paraffin-embedded sections, with appropriate negative controls, as described previously[14]. Human control abdominal and AAA sections were surgical samples procured by Dr. John Curci at Vanderbilt Medical Center, TN. The sample details are:

Sample	Age	Sex
Control abdominal aorta -1	56	M
Control abdominal aorta -2	64	F
Control abdominal aorta -3	51	M
AAA-1	61	M
AAA-2	70	F
AAA-3	68	M

Western Blot Analyses:

Mouse Aortic Lysates: Abdominal aortic tissue lysates were extracted in radioimmunoprecipitation assay lysis buffer, and protein content was measured using a Bradford assay (Bio-Rad, Hercules, CA). Protein extracts (20–30 μg) were resolved by SDS-PAGE (6.0 or 7.5 % wt/vol) and transferred electrophoretically to PVDF membranes (Millipore). After blocking with non-dry fat milk (5 % wt/vol), membranes were probed with primary antibodies. The following antibodies were used (please see Major Resources Table in the Data Supplement): calpain-1 domain IV (Abcam, catalog No: ab39170), calpain-2 (Abcam, catalog No: ab39165), filamin A (Abcam, catalog No: ab76289), talin (Abcam, catalog No: ab71333), α-spectrin (Millipore, catalog No: MAB1622), ILK-1 (Cell Signaling, catalog No:3862), FAK (Cell Signaling, catalog No:3009), vinculin (Abcam, catalog No: ab73412), paxillin (Abcam, catalog No: ab32264), collagen (Abcam, catalog No: ab34710), elastin (Abcam, catalog No: ab217356), calnexin (Enzo, catalog No: ADI-SPA-860-F) and β-actin (Sigma-Aldrich, catalog No: A5441). Membranes were incubated with appropriate HRP-labeled secondary antibodies. Immune complexes were visualized by chemiluminescence (Pierce, Rockford, IL) and quantified using a Kodak Imager.

Human AAA Lysates: Human AAA tissue were fresh frozen surgical samples procured by Dr. Gerard Pasterkamp, at the University Medical Center Utrecht, Netherlands. As

controls, fresh frozen human infra-renal aortic tissues were purchased from the Tissue for Research Company, UK. These control tissues were collected within 3 h post-mortem from organ donors in a deidentified manner. The sample details are:

Sample	Age	Sex
Control abdominal aorta -1	80	M
Control abdominal aorta -2	61	F
Control abdominal aorta -3	58	M
Control abdominal aorta -4	64	M
Control abdominal aorta −5	75	F
Control abdominal aorta −6	63	M
AAA-1	71	M
AAA-2	64	M
AAA-3	73	M
AAA-4	76	M
AAA-5	59	M
AAA-6	68	F

Control and AAA tissue lysates were extracted and protein content was measured in a similar method to mouse aortic tissues. The calpain-2 and β-actin antibodies were used similarly as mentioned above.

Zymography

Aortic MMP activity was examined by in-gel zymography as described previously.[10] Abdominal aortic protein extracts (5 mg) were resolved under nonreducing conditions by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% wt/vol) polymerized in the presence of gelatin (2 mg/mL) to measure activities of MMP2 and MMP9. Gels were washed with Triton X-100 (2.5% vol/vol) and distilled water for 30 minutes each. Gels were then incubated overnight at 37°C in Tris buffer containing calcium chloride (5 mM) and sodium azide (0.02% w/w), pH 8.0. After incubation, gels were stained with Coomassie Brilliant Blue followed by destaining with acetic acid (7% vol/vol) and methanol (40% vol/vol). Gel images were captured using a Bio-Rad Imager, and unstained, translucent, digested regions represent areas of MMP activity.

qPCR analyses of aortic mRNA Abundance.

Total RNA was extracted from abdominal aortas using the fibrous tissue RNA extraction kit from Qiagen (catalog No: Z3100). RNA (500 ng) was reverse transcribed using the iScript cDNA synthesis kit (Cat #170–8891; Bio-Rad, Hercules, CA).[10, 19] Quantitative PCR was performed to quantify mRNA abundance using a SsoFas EvaGreen Supermix kit (Cat # 172–5203; Bio-Rad) on a Bio-Rad CFX96 cycler. mRNA abundances were calculated by normalization to internal control 18S rRNA. Non-template and no RT reactions were used as negative controls.[10, 19] The primers used are detailed in Table I in the Data Supplement.

Calpain Activity Assay:

Calpain enzyme activity was measured in human AAA and mouse aortic tissue lysates fluorimetrically using a commercially-available activity assay kit (Catalog No: K240–100; BioVision, Mountain View, CA) as described previously.[10] Aortic protein extracts (20 mg) were incubated with fluorogenic (4-trifluoromethyl coumarin labeled) calpain substrate for 60 min at 37°C. Mean fluorescence signals were measured using a microplate fluorescent plate reader (Spectramax M2; MolecularDevices, Sunnyvale, CA) per manufacturer's instructions.

Human Aortic SMCs, Adventitial Fibroblasts and Short Interfering RNA Knockdown

Human aortic SMCs and adventitial fibroblasts were purchased from Promo Cell (Catalog No: C-12533, C-12380) and cultured in growth medium (Promo Cell, Catalog No: C39262). The SMC and fibroblasts cell purity were verified using a SMC α-actin (1:100, Abcam catalog No: ab5694) and ERTR7 (1:100, Abcam catalog No: ab5694) antibodies by immunohistochemistry. Cells of passages 2–3 were used for the short interfering (si)RNA transfection study. SMCs were transfected with control siRNA or siRNA targeting human calpain-2 (Ambion-Silencer Select validated siRNA, ThermoFisher Scientific) sequences using RNAiMax lipofectamine transfection reagent (Life Technologies, ThermoFisher Scientific). After 48 h of transfection, cells were incubated with either vehicle or AngII (1 μM) for 24 h; then protein lysates were harvested for western blot analyses.

Rat Aortic SMCs and Short Interfering RNA Knockdown

Rat aortic SMCs were isolated and cultured as described previously.[20] Briefly, rat aortas were excised and dissected free of adventitia and fat. The vessels were further digested by collagenase type I (Worthington Biochemical, 1 mg/ml) and elastase type III (sigma 0.125 mg/ml) at 37°C in a CO_2 humidified chamber. Then, cell suspensions were centrifuged and the cell pellets were re suspended and grown in DMEM with 10% fetal bovine serum and penicillin and streptomycin (1%) in 5% CO_2 at 37°C. The SMC phenotype was determined as described above. Cells of passages 2–3 were used for the short interfering (si)RNA transfection study. SMCs were transfected with control siRNA or siRNA targeting rat calpain-2 or filamin A (Ambion - Silencer Select validated siRNA, ThermoFisher Scientific) sequences using RNAiMax lipofectamine transfection reagent (ThermoFisher Scientific). After 48 h of transfection, cells were treated with either vehicle or AngII (100 nM) for 24 h and then harvested for western blot analyses.

Mouse Aortic SMCs, Short Interfering RNA Knockdown and Immunofluorescence

Mouse aortic SMCs were purchased from Cell Biologics (Catalog No: C57–6080) and cultured in SMC growth medium (Cellbiologics, Catalog No: M2268) as described previously.[20] The SMC cell purity was determined as described above. Cells of passages 2–3 were used for the short interfering (si)RNA transfection study. SMCs were transfected with either control siRNA or siRNA targeting mouse filamin A, or calpain-2 (Ambion - Silencer Select validated siRNA, ThermoFisher Scientific) sequences using RNAiMax lipofectamine transfection reagent (ThermoFisher Scientific). After 48 h of transfection, cells were fixed, permeabilized, and collagen I immunostaining was performed using a rabbit anti-mouse

collagen I (1 μg/ml, catalog No. ab34710, Abcam). Fluorescent labelled goat anti-rabbit secondary antibody (1 μg/ml, catalog No. ab1754741, Abcam) was used to visualize positive immunostaining under fluorescent microscopy. The mean fluorescence intensity of 100 cells was measured by Image J software. Fluorescent Cy3-labelled clone 1A4 α-actin antibodies (Clone 1A4, Sigma-Aldrich, catalog No: C6198) were used to visualize cytoskeletal actin protein as described previously.[20]

Statistical Analyses

Data are represented as mean ± SEM. Statistical analyses were performed using SigmaPlot 13.0 (SYSTAT Software Inc., San Jose, CA, USA). Data were tested for normality and equal variance using the Shapiro-Wilk and Brown-Forsythe test. If data did not pass normality, then data were statistically transformed to natural log. Repeated measurement data were analyzed with SAS fitting a linear mixed model expressing the temporal trend in systolic blood pressure as a quadratic polynomial in time for each treatment. For two-group comparisons, un-paired Student's t test was performed for normally distributed and equally variant values, while Mann-Whitney Rank Sum test was performed for variables not passing normality or equal variance test. One or Two way ANOVA with Holm-Sidak post hoc analyses were performed for multiple-group and multiple-manipulation analysis. Fisher's exact probability test was used to determine differences between groups in the incidence of aneurysm formation and in mortality due to rupture. Values of $P<0.05$ were considered statistically significant.

RESULTS

Calpain-2 Protein and Activity are Increased in Human and Experimental AAAs.

In the present study, immunohistochemical staining of human AAA tissue sections showed a strong presence of calpain-2 protein compared to control abdominal aortic sections (Figure 1A–L). The immunohistochemical staining identified a strong distribution of calpain-2 in fibroblast rich adventitia, surrounding perivascular adipose tissue (PVAT), and infiltrating macrophages, with a weak staining in the intima and media of human AAAs. Similarly, immunohistochemical staining on AngII-induced mouse AAA sections detected a strong distribution of calpain-2 in infiltrated macrophages, surrounding PVAT, and fibroblast rich adventitia, but weak staining in the intima and media (Figure 1M–S). Furthermore, Western blot analyses showed a significant increase in calpain-2 protein in both human (Figure 1T,U) and AngII-induced AAAs compared to control tissues (Figure 1W,X). In addition, measurement of calpain activity using a fluorescent substrate of calpain, Ac-LLY-AFC, showed a significant increase in calpain activity in both human (Figure 1V) and experimental AAA tissues (Figure 1Y) compared to control tissues.

Generation of Inducible Calpain-2 Deficient Mice.

Since calpain-2 deficiency in mice is embryonically lethal[13], calpain-2 deficient LDL receptor −/− mice were generated using calpain-2 floxed (f/f) and Cre transgenic mice expressing a tamoxifen-inducible Cre recombinase under control of the ubiquitously expressed chicken β-actin promoter (ERT2 Cre+/0)[21, 22]. Both calpain-2 f/f and β-actin Cre transgenic mice were bred to an LDL receptor −/− background. Female calpain-2 f/f mice

were bred with male β-actin Cre transgenic mice to yield offspring homologous for the floxed allele and hemizygous for the Cre transgene (Cre+/0). Littermates that were homozygous for the floxed calpain-2 gene, but without the Cre transgene (Cre0/0), were used as control mice. Calpain-2 f/f and Cre genotypes were confirmed by PCR (Figure I in the Data Supplement). The basal level of aortic calpain-2 protein level in calpain-2 f/f Cre0/0 and +/0 mice were examined by western blot analyses. The basal calpain-2 protein level was equivalent between the 2 groups of mice (Figure II in the Data Supplement). To induce Cre recombinase activity, mice were injected with tamoxifen (25 mg/kg body weight) intraperitoneally for 5 consecutive days. Western blot analyses of aortic tissue protein showed complete depletion of capain-2 protein, without influencing calpain-1, in aortas from Cre+/0 mice (Figure 2A).

Inducible Depletion of Calpain-2 Reduced AngII-induced AAA in Mice.

To determine the role of calpain-2 in AngII-induced AAA, calpain-2 f/f mice that are Cre0/0 or +/0 were fed a saturated fat-enriched diet and infused with saline or AngII for 4 weeks. Inducible calpain-2 deficiency had no effect on body weight, total plasma cholesterol concentrations or systolic blood pressure (Table II in the Data Supplement). Calpain-2 deficiency significantly reduced AngII-induced aortic luminal dilation (Figure 2B,C) as measured by ultrasound (Diameter: Saline - Cre0/0: 0.80 ± 0.02 *vs* Cre+/0: 0.83 ± 0.01 P=NS, AngII - Cre0/0: 1.83 ± 0.10 *vs* Cre+/0: 1.10 ± 0.06, P<0.001; Two-way ANOVA). In addition, calpain-2 deficiency significantly reduced AngII-induced AAA formation (Figure 2D,E) as measured by external aortic width expansion (Mean width; Saline - Cre0/0: 0.81 ± 0.02 mm *vs* Cre+/0: 0.89 ± 0.01 mm, P=NS; AngII - Cre0/0: 2.05 ± 0.13 mm *vs* Cre+/0: 1.17 ± 0.09 mm, P<0.001; Two-way ANOVA). Furthermore, calpain-2 deficiency also significantly reduced AngII-induced AAA incidence (Figure 2F; defined as a >50% increase in suprarenal aorta width; Cre0/0: 91% *vs* Cre+/0: 30%, P<0.001; Fisher Exact Test) but had no significant effect on aortic rupture (Figure 2G; Cre0/0: 11% *vs* Cre+/0: 8%).

Histological staining of abdominal aortas using picrosirius red (Figure 2H–O) and Movat's Pentachrome (Figure 2P–W) staining revealed disruption of adventitial collagen (Figure 2L,M) and focal medial elastin layer (Figure 2T,U) in the Cre 0/0 group infused with AngII. However, the abdominal aortas from calpain-2 deficient mice (Cre+/0) showed preserved adventitial collagen (Figure 2N,O) and medial elastin layer (Figure 2 V,W,X) upon AngII infusion. In addition, calpain-2 deficiency had no effect on AngII-induced atherosclerotic lesion areas in aortic arches and thorax (Figure III in the Data Supplement) and ascending aortic expansion (Figure IV in the Data Supplement).

Calpain-2 Deficiency in Adipocytes Had no Influence on AngII-induced AAA Formation in Mice.

In mice, evidences suggest that obesity-accelerated perivascular inflammation play a critical role in acceleration of AngII-induced AAA formation[23]. Further, human AAA tissue characterization studies showed an accelerated inflammation in the surrounding periaortic adipose tissue. Based on the strong calpain-2 positive staining observed in both human and AngII-induced AAAs, next we sought to determine the contribution of adipocyte-derived calpain-2 in the development of AngII-induced AAAs. Adipocyte-specific calpain-2

deficient LDL receptor −/− mice were generated using calpain-2 f/f and Cre transgenic mice expressing Cre recombinase under control of the adipocyte-specific adiponectin (Adipoq) promoter. Both calpain-2 f/f and Adipoq Cre transgenic mice were bred to an LDL receptor −/− background. Female calpain-2 f/f mice were bred with male Adipoq Cre transgenic mice to yield offspring homologous for the floxed allele and hemizygous for the Cre transgene (Cre+/0). Littermates that were homozygous for the floxed calpain-2 gene, but without the Cre transgene (Cre0/0), were used as control mice. Calpain-2 f/f and Cre genotypes were confirmed by PCR (Figure V in the Data Supplement). Western blot analyses of various fat pads (retroperitoneal, brown adipose, periaortic adipose) and aorta showed a strong reduction of calpain-2 in adipose tissue with intact calpain-2 in the aorta (Figure VIA in the Data Supplement).

To determine the role of adipocyte-calpain-2 in AngII-induced AAA, calpain-2 f/f mice that are Adipoq Cre0/0 or +/0 were fed a saturated fat-enriched diet and infused with saline or AngII for 4 weeks. Adipocyte-specific calpain-2 deficiency had no effect on body weight or total plasma cholesterol concentrations (Table III in the Data Supplement). Calpain-2 deficiency in adipocytes had no effect on AngII-induced aortic luminal dilation (Figure VIB in the Data Supplement), and external aortic width expansion (Figure VIC in the Data Supplement; P=NS; Two-way ANOVA). Furthermore, calpain-2 deficiency in adipocytes showed no difference on AngII-induced AAA incidence and aortic rupture (Figure VID,E in the Data Supplement). In addition, calpain-2 deficiency in adipocytes had no effect on AngII-induced atherosclerotic lesion areas in aortic arches and ascending aortic expansion (Figure VII in the Data Supplement).

Inducible Depletion of Calpain-2 in Col1a2 Positive Mesenchymal Cells Reduced AngII-induced AAA in Mice.

Previous studies demonstrated that tamoxifen mediated induction of Col1a2 Cre-recombinase (Col1a2-ERT2) resulted in activation of Cre recombinase in fibrogenic mesenchymal cells (aortic vascular SMCs and fibroblasts)[6, 24]. Since many cell-specific promoters have promiscuous expression that led to broader expression than defined by nomenclature, first, we determined the distribution of Col1a2-ERT2 Cre recombinase by breeding these transgenic mice to ROSA26^{LacZ} reporter mice. Whole aortic tissue showed presence of β-galactosidase (β-gal) activity in Col1a2-ERT2 Cre+/0 mice (Figure 3A), whereas no positive activity was detected in non-transgenic littermates (Cre0/0). Furthermore, to determine distribution of Col1a2-ERT2 Cre-lacZ in the aorta, we serially sectioned the aorta and found that β-gal is strongly distributed in the SMC rich aortic media and fibroblast rich adventitia throughout the aorta (Figure 3B–E). In addition to aorta, presence of β-gal in fibroblast and SMC rich organs (e.g. bladder, intestine, lung, skin etc.) and absence of β-gal in non-fibrotic organs such as brain, thymus (Figure VIII in the Data Supplement) confirm the specificity of Col1a2-ERT2 Cre.

To develop fibrogenic mesenchymal cell specific- calpain-2 deficient mice, both calpain-2 f/f and Col1a2-ERT2 Cre transgenic mice were bred to an LDL receptor −/− background. Female calpain-2 f/f mice were bred with male Col1a2-ERT2 Cre transgenic mice to yield offspring homologous for the floxed allele and hemizygous for the Cre transgene (Cre+/0).

Littermates that were homozygous for the floxed calpain-2 gene, but without the Cre transgene (Cre0/0), were used as control mice. Calpain-2 f/f and Cre genotypes were confirmed by PCR (Figure IX in the Data Supplement). To induce Cre recombinase activity, mice were injected with tamoxifen (25 mg/kg body weight) intraperitoneally for 5 consecutive days. Western blot analyses of SMC-rich aortic medial or fibroblast rich aortic adventitial tissue protein confirmed a dramatic reduction of capain-2 protein, in Cre+/0 mice (Figure 3F,G).

To determine the role of mesenchymal cell-derived calpain-2 in AngII-induced AAA, calpain-2 f/f mice that are Cre0/0 or +/0 were fed a saturated fat-enriched diet and infused with saline or AngII for 4 weeks. Inducible calpain-2 deficiency in mesenchymal cells had no effect on body weight, total plasma cholesterol concentrations or systolic blood pressure (Table IV in the Data Supplement). Calpain-2 deficiency significantly reduced AngII-induced aortic luminal dilation (Figure 4A,B) as measured by ultrasound ($P<0.05$; Two-way ANOVA). In addition, calpain-2 deficiency significantly reduced AngII-induced AAA formation (Figure 4C,D) as measured by external aortic width expansion ($P<0.05$; Two-way ANOVA). Furthermore, calpain-2 deficiency also significantly reduced AngII-induced AAA incidence (Figure 4 E; $P<0.001$; Fisher Exact Test) but had no significant effect on aortic rupture (Figure 4F; Cre0/0: 39% *vs* Cre+/0: 15%, $P=0.09$; Fisher Exact Test). Histological staining of abdominal aortas using Movat's Pentachrome staining (Figure 4O–V) revealed occurrence of focal elastin layer disruption (Figure 4S,T) in the Cre 0/0 group infused with AngII. However, abdominal aortas from calpain-2 deficient mice (Cre+/0) showed preserved medial elastin layers (Figure 4U,V,W) and adventitial collagen (Figure 4M,N) during AngII infusion compared to Cre0/0 group (Figure 4K,L). In addition, calpain-2 deficiency in fibrogenic mesenchymal cells had no effect on AngII-induced atherosclerotic lesion areas in aortic arches and ascending aortic expansion (Figure X in the Data Supplement).

Fragmentation of Cytoskeletal Structural Proteins are Increased in Human AAAs.

In AAA tissues, structural integrity of aortic medial tissue is highly disrupted[2, 3] and many studies have focused extensively on extracellular matrix (ECM) degradation as a part of the mechanism.[25–27] Currently, in AAAs, no reports are available on cytoskeletal structural proteins which maintain cellular structural integrity by connecting contractile proteins, α-actin and myosin, with the ECM. Filamin A, an actin binding protein, and talin, an integrin binding protein, contribute to organization and stability of the actin cytoskeleton, integrate cellular signaling cascades, and regulate cellular functions including adhesion and motility[28–30]. To examine whether human AAA is associated with fragmented cytoskeletal proteins, tissue lysates from human AAA and control aortas were analyzed for filamin A and talin fragmentation by Western blot using antibodies specific for their C-terminal domain. C-terminal fragmentation of filamin A and talin (Figure 5A,B) were significantly increased in human AAA compared to control aortic tissue, without influencing intact filamin A and talin. These data clearly demonstrate that increased cytoskeletal structural linker protein fragmentation is strongly associated with human AAAs.

Calpain-2 deficiency Suppressed AngII-induced Cytoskeletal Structural Protein Fragmentation in the Abdominal Aorta.

Calpain-2 protease is well known to regulate and target multiple proteins involved in various cellular functions including cytoskeletal structural organization as its substrate. Under AAA conditions, studies have extensively focused on the mechanisms underlying ECM degradation. Interestingly, no studies have explored the functional role of cytoskeletal structural linker proteins that connect contractile proteins, α-actin and myosin, with ECM. Based on the fact that calpain is the only known intracellular protease that targets cytoskeletal structural proteins and our current observations highlighting the increased fragmentation of cytoskeletal protein filamin A and talin (Figure 5A,B), next we examined if calpain-2 regulates cytoskeletal structural protein integrity during AAA development in mice. First, we tested whether AngII infusion promoted cytoskeletal structural protein fragmentation in aortas of mice. Calpain-2 f/f mice that were β-actin Cre0/0 or +/0 were fed a saturated fat-enriched diet and infused with either saline or AngII for 7 days. Western blot analyses using a calpain-2 specific antibody demonstrated that AngII-infusion increased calpain-2 protein abundance in calpain-2 f/f Cre 0/0 mice and none in Cre+/0 aortas as expected, with further phenotypic analysis confirming the deficient genotype (Figure 5C). Similarly, Western blotting using a calpain-1 specific antibody showed that AngII infusion increased calpain-1 protein abundance similarly in both Cre0/0 and +/0 mice with no further compensatory increase in the absence of calpain-2 (Figure 5C). Calpastatin, an endogenous inhibitor of calpain-1 and −2, is upregulated equivalently in both Cre0/0 and +/0 mice aortas during AngII infusion (Figure 5C). In addition, calpain-2 deficiency significantly suppressed AngII-induced calpain activity in the abdominal aorta (Figure XI in the Data Supplement). Similar in human AAAs, AngII infusion significantly promoted fragmentation of cytoskeletal proteins, filamin A, talin and spectrin, in the abdominal aorta of mice (Figure 5D–F). Interestingly, calpain-2 deficiency completely prevented the effect of AngII-induced fragmentation on filamin A, talin and spectrin. The observed bands of fragmented filamin and talin were further validated using rat aortic SMCs transfected with filamin A or talin siRNA and incubated with either saline or AngII. As expected, silencing of filamin A or talin resulted in a strong reduction of total filamin or talin protein along with a strong reduction or complete absence of fragmented proteins (Figure XII in the Data Supplement). In addition, calpain-2 deficiency also significantly prevented AngII-induced suppression of integrin linked kinase (ILK-1; Figure 5D,F), which plays an essential role in connecting the cytoplasmic tail of β subunits of integrins to the actin cytoskeleton, and regulating actin polymerization[31]. However, calpain-2 deficiency had no influence on AngII-induced focal adhesion kinase protein (Figure XIII in the Data Supplement). In addition, protein abundance of other cytoskeletal proteins such as vinculin and paxillin were not influenced by either AngII or calpain-2 deficiency in the aorta (Figure XIII in the Data Supplement). Furthermore, to understand the effect of calpain-2 deficiency on aortic extracellular matrix proteins, collagen and elastin, we performed Western analyses using abdominal aortic tissue lysates from Calpain-2 f/f β-actin Cre0/0 and Cre+/0 mice. AngII infusion for 7 days showed no change on collagen I protein in Cre0/0 groups; whereas it showed a significant increase in collagen I protein in Cre+/0 group (Figure 5 G,H). Similarly, qPCR analyses of aortic mRNA also revealed that calpain-2 deficiency significantly upregulated AngII-induced collagen I mRNA abundance compared to AngII-infused Cre0/0 group and saline

controls (Figure 5I). Calpain-2 deficiency had no influence on elastin protein in AngII-infused aortas (Figure 5 G,H). In addition, in-gel zymographic analyses of aortic lysates revealed that calpain-2 deficiency had no influence on AngII-induced activation of matrix metalloproteinases, MMP −2, and −9 (Figure XIV in the Data Supplement).

Consistent with the aortic tissue, siRNA mediated silencing of calpain-2 in human aortic adventitial fibroblasts or SMCs (Figure XVA,C in the Data Supplement) also prevented AngII-induced filamin A (Figure XVB,D in the Data Supplement) fragmentation which further confirms the contribution of calpain-2 in promoting fragmentation of cytoskeletal structural proteins, e.g. filamin A, in vascular wall cells.

Silencing of Filamin A or Talin Decreased ECM Protein, Collagen I in Aortic SMCs.

Cytoskeletal structural proteins act as linker proteins that hold contractile filaments, actin or myosin, intact with extracellular matrix proteins[4, 5, 32, 33]. In order to understand and demonstrate whether loss of cytoskeletal proteins had any influence on ECM proteins, we tested the effect of siRNA mediated silencing of filamin A on ECM proteins, collagen and elastin.

Based on observed similar filamin A fragmentation in both aortic SMCs and adventitial fibroblasts, we utilized SMCs for subsequent gene silencing studies. In addition, due to lack of availability of cells of human aortic origin, we performed gene silencing studies using rat aortic SMCs. In consistent with human aortic cells, siRNA mediated silencing of calpain-2 in rat aortic SMCs also prevented AngII-induced filamin A (Figure XVE,F in the Data Supplement) fragmentation. Transfection of rat aortic SMCs with filamin A siRNA for 48 h showed a significant reduction of filamin A (50%) protein compared to control siRNA transfected cells. Western blot analyses using antibodies specific against collagen I or elastin demonstrated that silencing of either filamin A (Figure XVG,H in the Data Supplement) strongly suppressed collagen I protein (Figure XVG,H in the Data Supplement) in aortic SMCs; whereas it had no effect on elastin protein (Figure XVG,H in the Data Supplement). Similarly, in mouse aortic SMCs transfected with filamin A siRNA, immunofluorescent staining using antibodies specific against collagen I revealed a strong reduction of collagen protein (Figure XVI and XVII in the Data Supplement).

Calpain-2 Deficiency Suppressed Rupture of Established AAAs.

Several studies have focused on ECM degradation[25] by extracellular proteases such as matrix metalloproteinases (MMPs)[26, 34] and cathepsins[27, 35, 36] as a mechanism; however, presently, no reports prove that inhibition of these proteases are beneficial in limiting AAA progression. Studies have demonstrated that prolonged infusion of AngII to hyperlipidemic mice resulted in progressive dilation of the aortic lumen,[37] which suggest that tamoxifen-inducible deletion of calpain-2 after an AAA formed from AngII infusion may be used to assess the effect of calpain-2 deficiency on AAA progression.

To examine whether calpain-2 deficiency had any influence on progression of established AAAs, a set of calpain-2 f/f β-actin Cre0/0 and Cre+/0 mice were infused with either saline or AngII for 28 days. The ultrasound measurement of aortic luminal dilation were performed on day 0 and 21 to ensure development of AAAs (Figure 6 A,B,C). On day 23, the mice

were injected with tamoxifen for 5 consecutive days to activate the Cre recombinase (Figure 6A). Then mice were continuously infused with either saline or AngII for another 8 weeks and aortic luminal dilation was periodically measured at days 42 and 70 of infusion (Figure 6D,E). On day 84, mice were terminated and aortas were examined. Inducible depletion of calpain-2 did not influence progressive lumen dilation (Figure 6F,G) and maximal ex-vivo aortic width expansion of AngII-induced established AAAs (Figure 6H,I) and AAA incidence (Figure 6J). Interestingly, inducible depletion of calpain-2 significantly suppressed mortality induced by aortic rupture (Figure 6K) and improved survival of calpain-2 deficient mice compared to controls from AngII-induced aortic rupture mediated death (Figure 6L).

DISCUSSION

In the present study, first we showed the clinical relevance of calpain-2 to AAA by demonstrating presence of increased calpain-2 protein and activity in human AAA tissues. In addition, we demonstrated that human AAAs are associated with increased cytoskeletal structural protein fragmentation. Furthermore, to determine the contribution of calpain-2 in AAA formation and cytoskeletal structural proteins in the aorta, we generated LDL receptor −/− mice with tamoxifen-inducible whole body calpain-2 deficiency using calpain-2 floxed and transgenic mice that express Cre recombinase under control of the ubiquitous promoter, chicken β-actin. Using this unique calpain-2 deficient mouse model, we examined the role of calpain-2 in AngII-induced AAAs. Here, we demonstrated that inducible depletion of calpain-2 in adult mice significantly reduced AngII-induced AAA formation. The beneficial effect of calpain-2 deficiency on AngII-induced AAA was associated with strong reduction of cytoskeletal structural protein destruction in the abdominal aorta of mice. Furthermore, using tamoxifen-inducible FMSC (including SMCs and fibroblasts) and adipocyte-specific calpain-2 deficient mice, we demonstrated that calpain-2 derived from vessel wall cells not from surrounding PVAT contributes to AngII-induced AAA development. In addition, in cultured aortic vascular cells, we demonstrated that silencing of cytoskeletal structural protein, filamin A, significantly reduced abundance of collagen protein which supports a functional role of cytoskeletal structural proteins for ECM protein integrity.

Whole body inducible depletion of calpain-2 significantly reduced AngII-induced AAA. Our study clearly demonstrates that depletion of calpain-2 is sufficient enough to significantly suppress AngII-induced calpain activity in the abdominal aorta. In our earlier studies, we showed that deficiency of calpain-1, another major isoform of calpain, or calpain-2 deficiency in leukocytes had no effect on AngII-induced AAA.[11, 12] Our current observation clearly demonstrates that calpain-2 derived from vessel wall cells (SMCs/fibroblasts) plays a critical role in AngII-induced AAA formation in mice. Fibrogenic mesenchymal cells that including fibroblasts, specialized myofibroblasts, synthetic aortic SMCs, which in common share the phenotype of expressing Col1, play a critical role in AngII-induced aortic fibrosis and ECM remodeling.[6] A series of studies highlighted that these fibrogenic mesenchymal cells regulate ECM remodeling through MMP activation,[38] fibronectin production,[39] vascular inflammation via RelA/NF-kB signaling and cytokine production.[6] To our knowledge, this is the first study to report the functional contribution of intracellular protease, calpain-2 in regulation of ECM in fibrogenic mesenchymal cells, during AAA development. In support, in our study, immunohistochemical staining showed a strong

distribution of calpain-2 in fibroblast rich adventitia and a weak distribution in SMC rich media in addition to infiltrating leukocytes and surrounding PVAT of human and AngII-induced AAA tissues. Our earlier study using leukocyte-specific calpain-2 deficient mice,[12] and our current observation using adipocyte-specific clapin-2 deficient mice, clearly demonstrates that calpain-2 derived from fibrogenic mesenchymal cells, but not from infiltrating leukocytes or surrounding PVAT, plays a critical role in AngII-induced AAA formation in mice.

AngII-induced AAA in hyperlipidemic mice is preceded by aortic dissection, which was characterized by presence of hematoma and thrombi mainly constrained in the adventitial site of the abdominal aorta.[40] Although it is not clear, dissection could be due to a defective balance between promotion of adventitial fibrosis by AngII versus its ability on promoting ECM destruction by activating MMPs.[40] Interestingly, in our study, depletion of calpain-2 completely protected mice from developing AngII-induced aortic dissection, which could be due to suppression of ECM proteins especially collagen regulation by calpain-2 possibly via destruction of cytoskeletal structural protein organization. Our current study is the first to report presence of cytoskeletal structural protein fragmentation in both human and experimental AAAs. Cytoskeletal structural proteins act as linker proteins that hold contractile filaments, actin or myosin, intact with extracellular matrix proteins.[4, 5, 32, 33] Loss of these structural protein may lead to destruction of cell structural integrity. Calpain-2 deficiency or silencing completely prevented AngII-induced fragmentation of filamin A and talin in mice, and in cultured aortic adventitial fibroblasts and SMCs. In addition, gene silencing of filamin A in cultured aortic SMCs led to complete loss of ECM protein collagen, but had no effect on elastin. However, the differential regulation of collagen and elastin by cytoskeletal proteins in cultured aortic SMCs needs further investigation. The possible mechanism underlying the dramatic reduction of collagen I protein in filamin A silenced aortic SMCs could be due to defective organization of collagen fibers and/or suppression of collagen production. In support, in a wound healing model, using skin fibroblasts from filamin A conditional knockout mice, Mohammadi et al. suggested that filamin A provides instructional cues that regulate maintenance of tension in collagen fibers during remodeling.[41] In addition, using cultured wild type or filamin A knockdown 3T3 fibroblasts, Mezawa et al. demonstrated that filamin A expression strongly affects the abundance of collagen on the cell surface and route of collagen degradation possibly via its ability to bind actin filaments which is important in the assembly and retention of pericellular collagen.[42] Also, our studies using inducible whole body calpain-2 deficient mice clearly showed a significant increase in aortic collagen mRNA and protein during AngII infusion, and significantly suppressed rupture of established AAAs. The data from in vitro and in vivo studies suggest that calpain-2 mediates AngII-induced cytoskeletal structural protein destruction, which in turn may promote ECM disorganization and disruption of aortic structural integrity. These observations clearly indicate that cytoskeletal structural proteins play a critical role in maintaining ECM proteins. Further studies are warranted to understand mechanisms by which cytoskeletal structural proteins help maintain stability of ECM.

In the AAA progression study, calpain-2 depletion suppressed AAA rupture but had no effect on AAA growth during continuous AngII infusion. As reported by several earlier

studies and further confirmed in our present study, AngII-induced AAA is strongly associated with elastin and collagen degradation in the aortic media and adventitia.[37] The reduction of ECM proteins makes the aorta more susceptible to further dilation and rupture upon continuous and prolonged infusion of AngII. One possible explanation for the observed beneficial effect on AAA rupture not on AAA growth could be due to either stabilized or increased collagen I in the aorta post calpain-2 depletion during continuous and prolonged infusion of AngII-induced AAA progression. However, further studies are warranted to test and confirm the production or stabilization of collagen 1 in the aorta during AAA progression post calpain-2 depletion.

One limitation of our present study is the lack of corn oil control groups in our different tamoxifen-inducible calpain-2 depletion mice studies. Intraperitoneal injection of corn oil has been shown to induce mild chronic peritoneal inflammation in mice compared to other oil controls such as peanut or mineral oil by depleting residential peritoneal macrophages.[43] The oil injection-induced peritoneal inflammation may in turn promote periaortic vascular inflammation and accelerate AngII-induced AAA formation in mice. However, our current study utilizing adipocyte-specific calpain-2 deficient mice and our earlier published study utilizing leukocyte-specific calpain-2 deficient mice[12] clearly demonstrated that (i) AngII-induced calpain-2 activation is associated with cytoskeletal structural protein and ECM destruction in the aorta, and (ii) calpain-2 derived from either adipocytes or leukocytes had no influence on AngII-induced AAA formation. Therefore, inclusion of corn oil control groups in tamoxifen-inducible whole body or fibrogenic mesenchymal calpain-2 deficient mice would be helpful to understand the contribution of corn oil injection-induced peritoneal inflammation in AngII-induced cytoskeletal structural protein and ECM destruction, and AAA formation in mice with or without calpain-2 deficiency.

One interesting observation in our present study is the observed differences in aortic rupture rate in the littermate calpain-2 floxed (Cre0/0) controls in the inducible whole body (β-actin – 10%) and fibrogenic mesenchymal cell-specific (Col1α2 – 39%) calpain-2 mice aneurysmal studies. As stated in the methods sections, the Col1α2 cre mice originated from a mixed C57BL6 × DBA/2 background compared to β-actin Cre+/0 mice which is of C57BL/6 background. Both mice were backcrossed to C57BL/6J into 8–10 times. One speculative explanation for the observed higher rupture rate of 39% in Col1α2 Cre0/0 floxed controls over 10 % rupture rate of to β-actin Cre0/0 floxed controls could be due to the difference in genetic background between the 2 groups of mice (C57BL/6 × DBA/2 vs C57BL/6). However, currently there are no reports or literature available on AngII-induced aortic rupture rate in mice with different genetic backgrounds. Further studies are warranted to address the discrepancies in AngII-induced aortic rupture rate between the different genetic background in mice.

Currently, it is not clear by which mechanism calpain-2 mediates or contributes to cytoskeletal structural protein destruction. Calpain cleavage site prediction analyses using the online calpain cleavage site predictor software (CaMPDB calpain.org) revealed various strong cleavage sites in structure of filamin A.[44] Filamin A has a calpain binding site at Ser2152 residue in the H1 region.[45] However, further studies are warranted to understand whether calpain-2 mediates its effect on these structural proteins through direct binding on

these predicted sites or via an indirect mechanism. Utilizing cells that express structural protein mutations at potential calpain-2 binding sites, our future studies will delineate the functional role of calpain-2 in cytoskeletal structural protein destruction.

In the clinical setting, therapies are only initiated in patients with established AAAs. The observed beneficial effect of calpain-2 depletion on AAA formation in the initiation study and on AAA rupture in the progression study suggests that pharmacological inhibition of calpain-2 may be clinically helpful either to slow progression of AAA at an early stage or to suppress / stabilize AAA rupture.

In summary, we demonstrated that inducible depletion of calpain-2 decreased AAA formation, and rupture of established AAAs, which was associated with reduced cytoskeletal structural protein fragmentation and increased collagen matrix protein in mice (Graphical abstract). These results suggest that inhibition of calpain-2 may offer a new therapeutic target to reduce AAAs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms:

AngII	angiotensin II
AAA	abdominal aortic aneurysm
LDL	low-density lipoprotein
ECM	extracellular matrix
SMC	smooth muscle cell

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HIGHLIGHTS

- Human and experimental AAAs are associated with increased calpain-2 protein and activity, and increased cytoskeletal structural protein fragmentation.
- Inducible depletion of calpain-2 in mice decreases AAA formation, which is associated with reduced fragmentation of cytoskeletal structural proteins.
- Targeted inhibition of calpain-2 may offer a new therapeutic direction to stabilize and suppress AAA rupture.

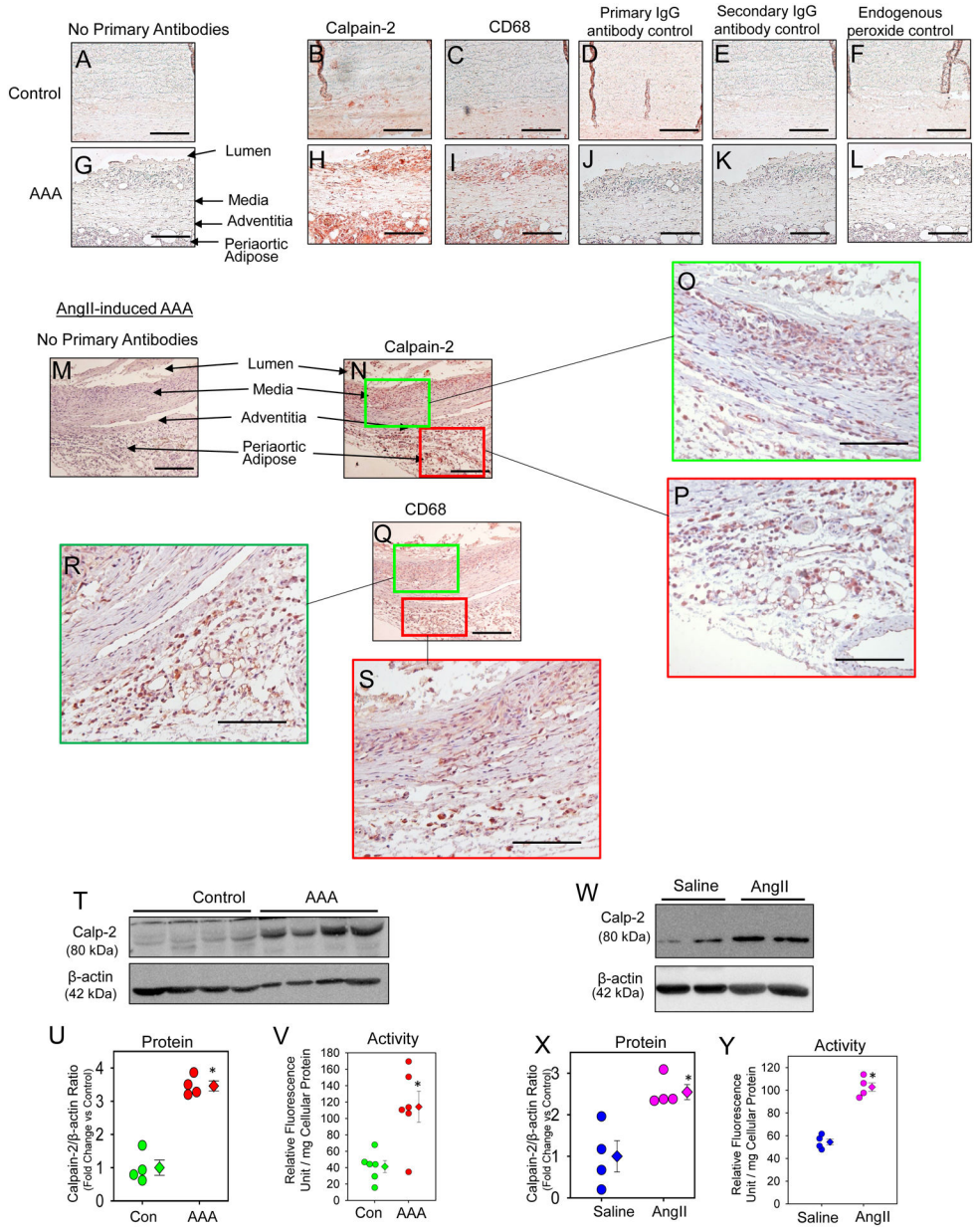

Figure 1. Calpain-2 protein and activity are increased in human and experimental AAAs.

Serial cross-sections from human AAA and control abdominal aortic tissue sections (**A, G**) were immunostained with anti-calpain-2 (**B, H**) and CD68 (**C, I**). Red color indicates positive immunostaining. Representative control and AAA tissue sections stained with rat and rabbit primary IgG iso-type control antibodies (**D, J**), secondary antibodies (Rabbit anti-rat or Goat anti-rabbit- **E,K**) and endogenous peroxide (no antibodies - **F,L**) control. Serial cross-sections from AngII-induced AAA tissue sections were immunostained with anti-calpain-2 (**M,N,O,P**) and CD68 (**Q,R,S**). Nuclei were counterstained with hematoxylin. Scale bars correspond to 50 μm (200x magnification). Calpain-2 protein detection in human control abdominal aorta and AAA tissues (n=4) (**T,U**). Calpain activity (**V**) in human control and AAA tissues (n=6). Calpain-2 and β-actin protein in abdominal aortas from saline and

AngII-infused LDL receptor−/− mice (n=6) (**W,X**). Calpain activity (**Y**) in abdominal aortas from saline and AngII-infused LDL receptor−/− mice (n=4). * denotes P<0.05 when comparing control vs AAA or saline vs AngII infusion (Student's t test).

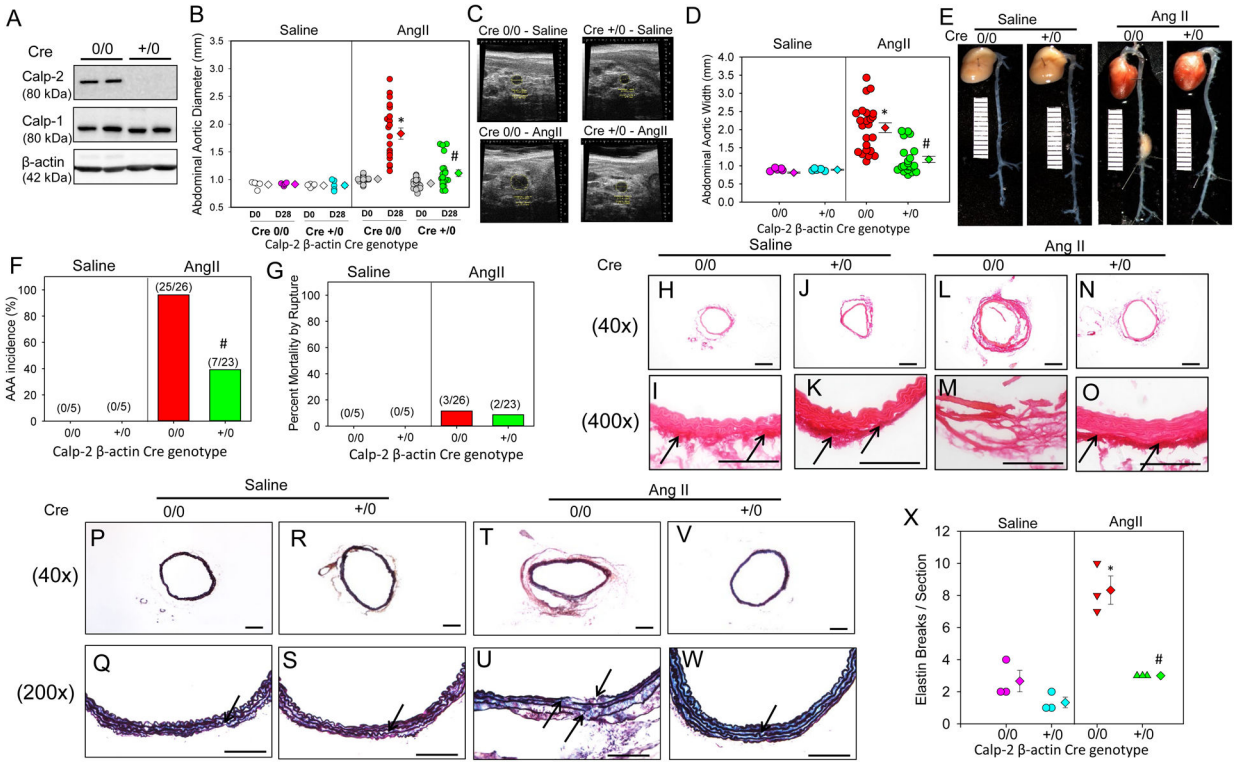

Figure 2. Calpain-2 deficiency significantly reduced AngII-induced AAAs.

A.Calpain-2 and β-actin protein were detected in aortic tissue lysates from calpain-2 f/f β-actin (ERT2) Cre0/0 and +/0 mice. **B.** Abdominal aortic luminal diameters were measured by ultrasound on day 0 and after 28 days of saline or AngII infusion (Saline n=5; AngII n=23–26). **C.** Ultrasound pictographs of suprarenal aortas of individual mice nearest to the mean of the group. **D.** Measurements of maximal external width of abdominal aortas (Saline n=5; AngII n=23–26). Pink (saline - Cre 0/0), teal (saline - Cre +/0), red (AngII - Cre 0/0) and green (AngII - Cre +/0) represent individual mice, diamonds represent means, and bars are SEMs. **E.** Representative images of aortas nearest the mean of each group. * denotes P<0.05 when comparing saline vs AngII infusion; # denotes P<0.05 when comparing Cre 0/0 vs Cre +/0 mice (Two-way ANOVA with Holm-Sidak post hoc analysis). **F.** The incidence of AAA (>50% increase in aortic width) in AngII-infused calpain-2 f/f mice that were either β-actin Cre 0/0 (red bar) or Cre +/0 (green bar). Statistical analyses were performed by Fisher Exact test (# denotes P<0.001 when comparing Cre 0/0 vs Cre +/0 mice). **G.** Mortality due to AAA rupture in AngII-infused calpain-2 f/f mice that were either β-actin Cre 0/0 or +/0. Red (Cre0/0) and green bar (Cre+/0). Representative suprarenal aortic tissue-sections from saline (**H-K;P-S**) and AngII (**L-O;T-W**) administered calpain-2 f/f mice that were β-actin Cre 0/0 and +/0 stained with Picrosirius red or Movat's Pentachrome. Arrows indicates medial breaks. Scale bars correspond to 50 μm. H,J, L,N,P,R, T and V = 40x; I,K,M and O = 400x; Q,S,U and W = 200x. **X.** Measurements of aortic medial break in the cross-sections of abdominal aortas (n=3 in each group). Pink (saline - Cre 0/0), teal (saline - Cre +/0), red (AngII - Cre 0/0) and green (AngII - Cre +/0) represent individual mice, diamonds represent means, and bars are SEMs. * denotes P<0.05

when comparing saline vs AngII infusion; # denotes P<0.05 when comparing Cre 0/0 vs Cre +/0 mice (Two-way ANOVA with Holm-Sidak post hoc analysis).

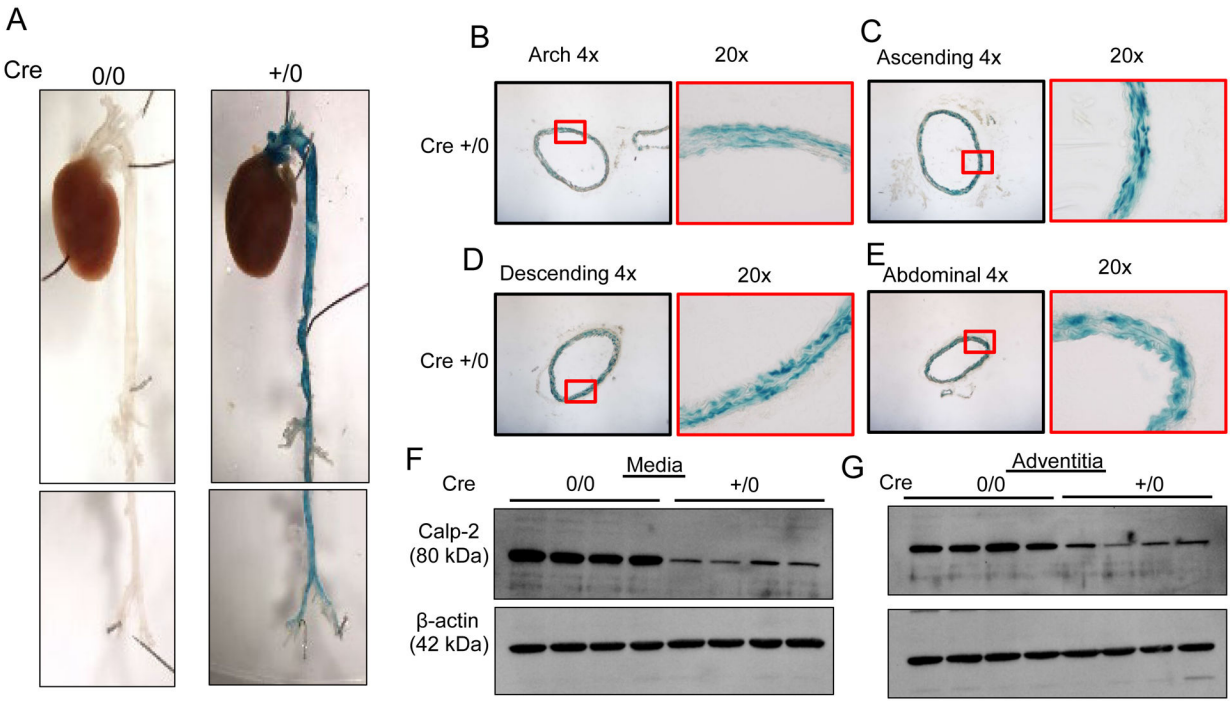

Figure 3. Distribution of Col1a2 positive mesenchymal cells in the aorta.

A. Representative ventral views of β-galactosidase (β-gal) activity in aortas from Col1a2 Cre positive (Cre+/0) and control (Cre0/0) mice. Blue color is positive staining for distribution of Cre excision. Cross-sections of arch (**B**), ascending (**C**), descending (**D**) and abdominal (**E**) aortas from Col1a2 Cre mice stained with X-gal. Calpain-2 and β-actin protein were detected in aortic medial (**F**) and adventitial (**G**) tissue lysates from calpain-2 f/f Col1a2 Cre 0/0 and +/0 mice.

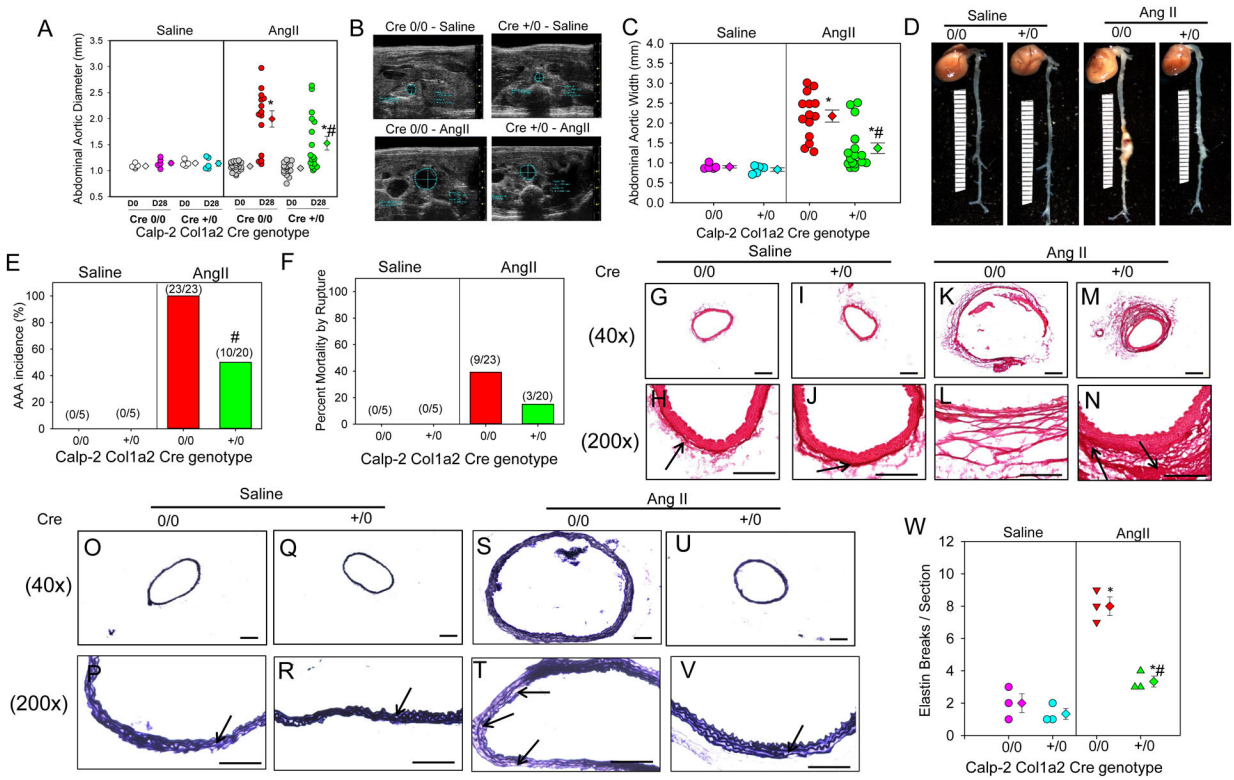

Figure 4. Calpain-2 deficiency in Col1a2 positive mesenchymal cells reduced AngII-induced AAA in Mice.

A. Abdominal aortic luminal diameters were measured by ultrasound on day 0 and after 28 days of saline or AngII infusion (Saline n=5; AngII n=20–23). **B.** Ultrasound pictographs of suprarenal aortas of individual mice nearest to the mean of the group. **C.** Measurements of maximal external width of abdominal aortas (Saline n=5; AngII n=20–23). Pink (saline - Cre 0/0), teal (saline - Cre +/0), red (AngII - Cre 0/0) and green (AngII - Cre +/0) represent individual mice, diamonds represent means, and bars are SEMs. * denotes P<0.05 when comparing saline vs AngII infusion; # denotes P<0.05 when comparing Cre 0/0 vs Cre +/0 mice (Two-way ANOVA with Holm-Sidak post hoc analysis). **D.** Representative images of aortas nearest the mean of each group. **E.** The incidence of AAA (>50% increase in aortic width) in AngII-infused calpain-2 f/f mice that were either Col1a2 Cre 0/0 (red bar) or Cre +/0 (green bar). Statistical analyses were performed by Fisher Exact test (# denotes P<0.001 when comparing Cre 0/0 vs Cre +/0 mice). **F.** Mortality due to AAA rupture in AngII-infused calpain-2 f/f mice that were either Col1a2 Cre 0/0 or +/0. Red (Cre0/0) and green bar (Cre+/0). Representative suprarenal aortic tissue-sections from saline (**G-J;O-R**) and AngII (**K-N;S-V**) administered calpain-2 f/f mice that were Col1a2 Cre 0/0 and +/0 stained with Picrosirius red or Movat's Pentachrome. Arrows indicates medial breaks. Scale bars correspond to 50 μm. G,I,K,M,O,Q,S and U = 40x; H,J,L,N,P,R,T and V = 200x. **W.** Measurements of aortic medial break in the cross-sections of abdominal aortas (n=3 in each group). Pink (saline - Cre 0/0), teal (saline - Cre +/0), red (AngII - Cre 0/0) and green (AngII - Cre +/0) represent individual mice, diamonds represent means, and bars are SEMs. * denotes P<0.05 when comparing saline vs AngII infusion; # denotes P<0.05 when comparing Cre 0/0 vs Cre +/0 mice (Two-way ANOVA with Holm-Sidak post hoc analysis).

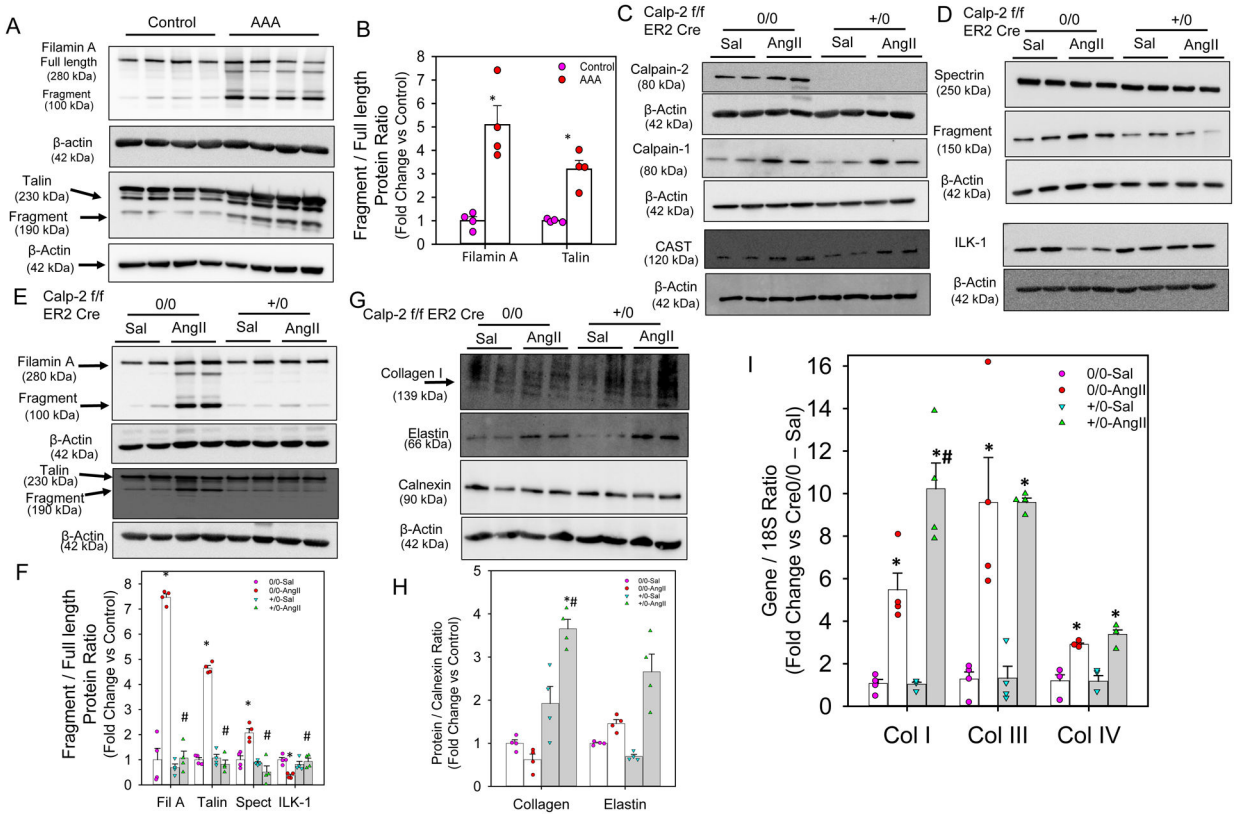

Figure 5. Fragmentation of cytoskeletal structural proteins is increased in human AAA tissues.

Fragmentation of cytoskeletal structural proteins is increased in human AAA tissues. A,B. Full length and fragments of filamin A and talin proteins were detected in human control abdominal aorta and AAA tissues (n=4) by Western blot. * denotes P<0.05 when comparing control vs AAA (Student's t test or Mann-Whitney Rank Sum test). **Calpain-2 deficiency significantly suppressed AngII-induced cytoskeletal structural protein fragmentation. C,D,E,F.** Calpain-2, calpain-1, full length and fragments of filamin A, talin and spectrin proteins, CAST and ILK-1 and β-actin proteins were detected in abdominal aortas from saline and AngII-infused calpain-2 f/f β-actin Cre0/0 and +/0 mice (n=4). **G,H.** Collagen I, elastin, calnexin and β-actin protein were detected in abdominal aortas from saline and AngII-infused calpain-2 f/f β-actin Cre0/0 and +/0 mice (n=4). **Calpain-2 deficiency significantly increased AngII-induced Collagen I mRNA abundance in the abdominal aorta. I.** mRNA abundance of Col I, III and IV genes in abdominal aortas from saline and AngII-infused calpain-2 f/f β-actin promoter Cre0/0 and +/0 LDL receptor−/− mice (n=4/group) were analyzed by qPCR. * denotes P<0.05 when comparing saline vs AngII infusion; # denotes P<0.05 when comparing Cre 0/0 vs Cre +/0 mice (Two-way ANOVA with Holm-Sidak post hoc analysis).

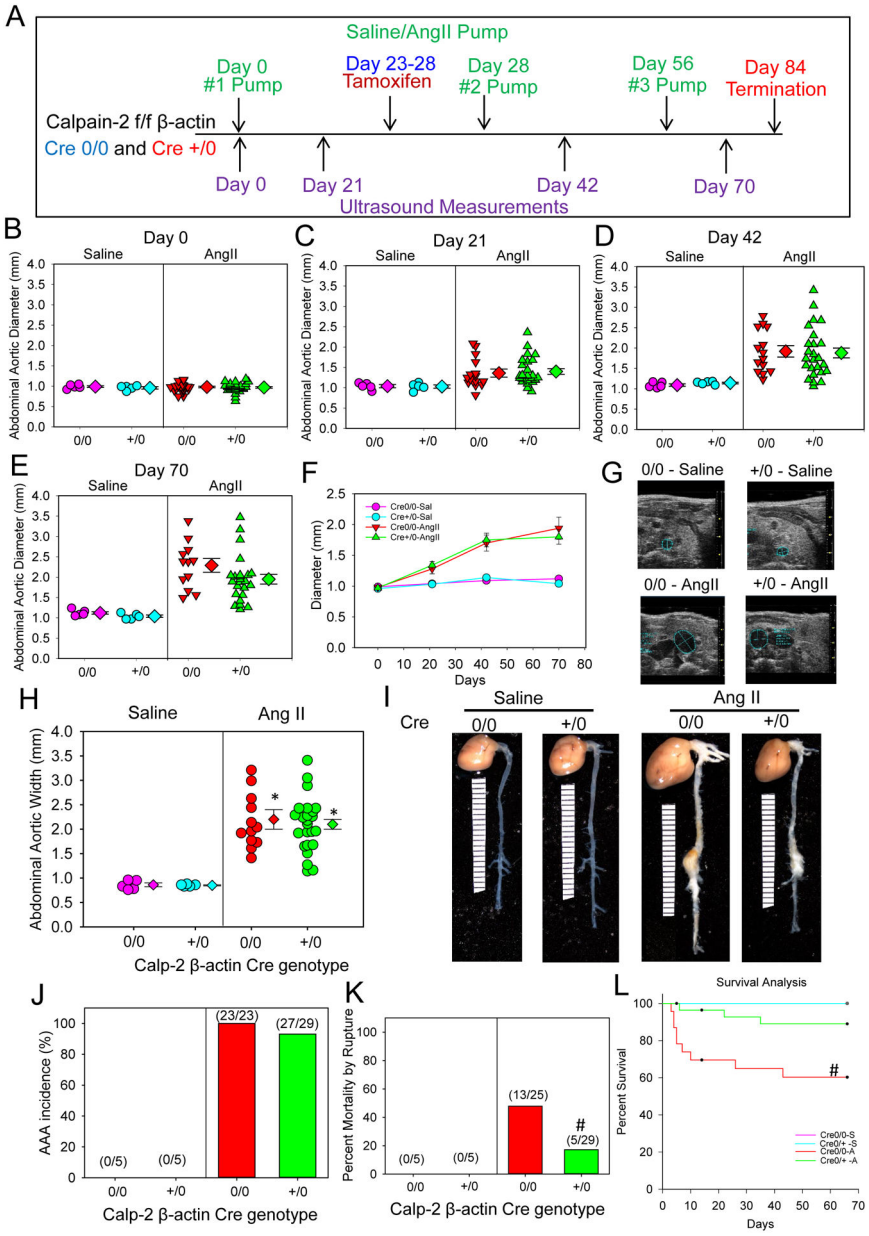

Figure 6. Inducible depletion of Calpain-2 deficiency significantly suppressed rupture of established AAAs.

A. Experimental design to study the effect of inducible-calpain-2 deficiency on progression of AngII-induced established AAAs. Abdominal aortic luminal diameters were measured by ultrasound on day 0 **(B)**, after 21**(C)**, 42 **(D)**, and 70 **(E)**, days of either saline or AngII infusion (Saline n=5; AngII n=23–29). **F.** Progressive luminal dilation over the course of 84 days of saline or AngII infusion. **G.** Representative images of ultrasound pictures of abdominal aortas of Cre 0/0 and +/0 mice infused with saline or AngII. **H.** Measurements of maximal external width of abdominal aortas (Saline n=5; AngII n=23–26). **I.** Representative images of aortas nearest the mean of each group. Pink (saline - Cre 0/0), teal (saline - Cre +/0), red (AngII - Cre 0/0) and green (AngII - Cre +/0) represent individual mice, diamonds represent means, and bars are SEMs. * denotes P<0.05 when comparing saline vs AngII

infusion; # denotes P<0.05 when comparing Cre 0/0 vs Cre +/0 mice (Two-way ANOVA with Holm-Sidak post hoc analysis). **J.** The incidence of AAA (>50% increase in aortic width) in AngII-infused calpain-2 f/f mice that were either β-actin Cre 0/0 (red bar) or Cre +/0 (green bar). **K.** Mortality due to AAA rupture in AngII-infused calpain-2 f/f mice that were either β-actin Cre 0/0 or +/0. Red (Cre0/0) and green bar (Cre+/0). **L.** Survival rate from the abdominal aortic rupture. Statistical analyses were performed by Fisher Exact test (# denotes P<0.001 when comparing Cre 0/0 vs Cre +/0 mice).