Online-Only Materials and Methods

Leukocyte Calpain Deficiency Reduces Angiotensin II-induced Inflammation and Atherosclerosis but not Abdominal Aortic Aneurysms in Mice

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Running Title: Leukocyte Calpain Deficiency Reduces Atherosclerosis

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MATERIALS AND METHODS

Mice

LDL receptor -/- (stock # 002207), LysM Cre+/0 (stock # 004781) and C57BL/6J (Stock# 000664) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice overexpressing calpastatin (CAST-Tg) driven by a cytomegalovirus promoter on a C57BL/6 background were generated originally in the laboratory of Dr. Laurent Baud.[1] Calpain-1 -/- mice on a C57BL/6 background were generated originally in the laboratory of Dr. Athar Chishti.[2, 3] Calpain-2 floxed (f/f) mice on a C57BL/6 background were originally generated in the laboratory of Dr. Takaomi Saido.[4] LDL receptor -/-, calpain-1 -/-, and calpain-2 f/f mice were backcrossed 10 times into a C57BL/6 background. CAST-Tg mice were backcrossed at least 9 generations into a C57BL/6 background.

To generate calpain-1 study mice in an LDL receptor -/- background, calpain-1 -/- males were mated to LDL receptor -/- females, and their offspring were bred to generate calpain-1 +/- males and females in the LDL receptor -/- genotype. Subsequent breeding generated relevant littermate controls of calpain-1 +/+ x LDL receptor -/- and calpain-1 -/- x LDL receptor -/- mice.

Both calpain-2f/f and LysM Cre+/0 mice were bred to an LDL receptor -/- background. Female calpain-2f/f mice were bred with male LysM Cre+/0 mice to yield mice homologous for the floxed allele 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.

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. All study procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee (Protocol # 2011-0907). This study followed the recommendations of The Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

Mouse Genotyping

Mouse genotypes were confirmed by PCR. DNA was isolated from tail snips or BM-derived cells using a Maxwell tissue DNA kit (Cat# AS1030, Promega, Madison, WI). CAST-Tg genotyping was performed using the following primers: 5'-GTTGGCTTAGGCTGCTTTTCGT-3' and 5'-CCAGACTCCGTGA CACCCCTT-3'. The resultant CAST-Tg PCR product was 518 base pairs (bp) and no product for non-transgenic mice. The IL-2 gene was used as an internal control for CAST-Tg genotyping using the following primers: 5'-CTAGGCCACAGAATTGAAAGAT CT and 5'-GTAGGTGGAAATTCTAGCATCATCC. The resultant product was 324 bp (Supplemental Figure S1). Calpain-1 genotyping used the following primers: 5'-TGCACTCTAGTTCTGAGG CT-3', 5'-AGAGTGCACGAACACCAGCTT-3', and 5'-TTAAGGGCCAGCTCATTCCT-3'. PCR of wild-type and disrupted alleles generated amplicons of 615 bp and 415 bp, respectively (Supplemental Figure S2). Calpain-2f/f genotyping used the following primers: 5'- ATAGCTCCTGTGTATCAG GCACAGAGCTGG- 3' and 5'- CTCTGGTCAGGTCTTAGTTCCCAGAGGATG - 3'. Resultant wild-type, and flox allele bands were 290, and 430 bp, respectively (Supplemental Figure S3). 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 as similar to CAST-Tg genotyping. LDL receptor genotyping was performed as described previously.[5]

Bone Marrow Transplantation

Recipient male LDL receptor -/- mice were provided drinking water containing sulfamethoxazole (4 µg/ml) for 1 week prior to irradiation, which was maintained for another 4 weeks after bone marrow transplantation. Recipient male LDL receptor -/- mice (8 weeks of age) were irradiated with 450 rads twice (total 900 Rads) at 3 hour from a cesium source and repopulated with bone marrow-derived cells harvested from male donor mice as described previously.[6] The mice that were used as donors include WT, CAST Tg, LDL receptor -/- mice that were either calpain-1 +/+ or -/-. BM-derived cells were resuspended and injected (5×10^6 cells/mouse) into tail veins of irradiated recipient mice. Four weeks after bone marrow transplantation, mice were fed a saturated fat-enriched diet and infused with saline or AngII for 4 weeks. Reconstitution of the transplanted bone marrow cells was determined by PCR on genomic DNA from bone marrow of recipient mice.

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.

AngII Infusion

After an initial week of high-fat diet feeding, mice were implanted with Alzet osmotic minipumps (model 2004, Durect Corporation, Cupertino, CA), subcutaneously into the right flank, and infused with saline or AngII (1,000 ng/kg/min, Bachem, Torrance, CA) continuously for a period of 7 or 28 days, as described previously.[7] Mice were maintained on high fat-enriched diet throughout the 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).[8] SBP was measured on 5 consecutive days prior to pump implantation, and during the last 5 days of AngII infusion.

Measurement of Blood and Plasma Components

Peripheral blood cell numbers were counted using a Hemavet 950 (Drew Scientific Inc, Dallas, TX). Plasma cholesterol concentrations were measured using a commercially available enzymatic kit (Wako Chemicals, Richmond, VA) as described previously.[5]

Quantification of Atherosclerosis and Abdominal Aortic Aneurysms

After saline perfusion 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 as lesion area, and percent lesion area on the intimal surface by en face analysis as described previously.[9, 10] Lesion areas were measured using Image-Pro Plus software (Media Cybernetics, Bethesda, MD) by direct visualization of lesions under a dissecting microscope. For aneurysm measurements, Abdominal aortic aneurysms (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]

Tissue Histology and Immunostaining

Ascending arch aortas were placed in optimal cutting temperature (OCT) compound and sectioned (10 µm thickness/section) in sets of 10 slides serially with 9 sections/slide by a cryostat.[12] One slide of each serial set was stained with hematoxylin and eosin to examine cellularity. Immunohistochemical staining was performed on aortic sections to detect calpain protein and specific cell types. Calpain-1 and -2 immunostaining were performed using the rabbit anti-mouse calpain-1 and -2 (10 µg/ml, catalog Nos. RP1-Calpain-1 and RP-2 Calpain-2; Triple Point biologics, Forest Grove, OR). The following antibodies were used to detect specific cell types: rat anti-mouse CD68 (1:200, catalog No. MCA1957; Serotec, Raleigh, NC) for macrophages; rabbit anti-mouse α-smooth muscle actin (2 µg/ml, catalog No: ab5694; Abcam, Cambridge, MA) for smooth muscle cells and rat anti-mouse ER-TR7 (1:200, catalog No: ab51824; Abcam, Cambridge, MA) for fibroblasts. Immunostaining was performed on formalin-fixed frozen sections, with appropriate negative controls, as described previously.[5, 13] The specificity of immunostaining was verified using appropriate negative controls (Supplemental Figure S7A, B). The endothelial cell purity was verified using biotin labeled CD31 antibody (1:100, catalog No: 55371 BD Pharmingen, San Jose, CA) by immunohistochemistry.

Macrophage Dil-AcLDL Uptake Assay

Dil-labeled Ac-LDL ((Life Technologies; catalog No: L35354) was used to trace the Ac-LDL uptake. Cellular uptake of Ac-LDL was measured using both fluorescence microscopy and flow cytometry as described previously[14] Bone marrow cells were harvested from the femurs of WT and CAST-Tg mice[6] and differentiated into BMDMs using RPMI media containing 10% FBS and 15% L929 Cells (mouse fibroblast) conditioned medium for 7 days.[15] The fresh media was provided every 48 h. On day 7, cells were trypsinized, counted, and replated in a volume of 1x 10^6 cells per well in a 6 well plate. After 24h, the cells were washed and maintained in RPMI+ 0.2% fatty acid free BSA for 24h. Then the cells were incubated with Dil Ac-LDL (25 µg/ml) for 24 h. Cells were washed with cold acid washing buffer (0.5 M glacial acetic acid, 150 mM sodium chloride, pH 2.5). For fluorescent microscopy analyses, the cells were fixed with formalin, and 10 fields were photographed using an inverted fluorescent microscope (Nikon Instruments, Melville, NY). The cells were counterstained with DAPI

for nuclei. The fluorescent cells were counted using Image Pro software (Image-Pro Cybernetics, Bethesda, MD). For flow cytometry, the cells were detached from the plate with EDTA (0.05mM) in PBS. The cells were counterstained with DAPI on ice for 10 min. The cells were then washed with FACS washing buffer (2 % FBS in PBS) and analyzed by FACS LSRII (BD, San Jose, CA). The data were quantified using CellQuest analytical software (BD, San Jose, CA).

Macrophage Adhesion Assay

Bone marrow cells were harvested from the femurs of WT and CAST-Tg mice[6] and differentiated into BMDMs using RPMI media containing 10% FBS and 15% L929 Cells (mouse fibroblast) conditioned medium for 7 days. The fresh media was provided every 48 h. On day 7, cells were trypsinized, counted, and re-suspended in RPMI media.[15] Then cells were labeled with calcein (3 µl/ml; Molecular Probes, catalog No: C-3099) at 37°C for 15 min. After labeling, the cells were washed twice to remove excess calcein. BMDMs were resuspended ($1x10^6$ in a volume of 50 µl/well) and then added to monolayer of cultured mouse aortic endothelial cells (CellBiolgics, catalog No C57-6052) that were pre-incubated with AngII (1 µM) overnight. The endothelial cell purity was verified using a biotin labeled CD31 antibody (1:100, catalog No: 55371 BD Pharmingen, San Jose, CA) by immunohistochemistry (Supplemental Figure S5). The cells were incubated at 37°C for 60 min and then gently washed to remove unbound BMDMs and the attached cells were fixed with 1% glutaraldehyde (Sigma; catalog No: G5882).[16] The cells were photographed from 10 fields at the power of 10x using a fluorescence microscope (Nikon, Melville, NY) and counted by two independent investigators in a blinded manner.

Macrophage Migration Assay

Migration assays were performed using transwells with 8.0-µm pore polycarbonate membrane inserts (Corning).[17] WT and CAST-Tg BMDMs were seeded ($1X10^6$ in a volume of 50µl media/well) on transwell filters, and lower chambers were filled with either control media or media containing MCP-1 (100 ng/mL). After a 6 h incubation at 37°C, cells were removed from the upper surface of inserts by scraping with Q-Tips. The membranes were fixed with 1% glutaraldehyde (Sigma; catalog No: G5882), stained with hematoxylin (Leica) and mounted on glass slides using glycerol gelatin. Hematoxylin-stained cells were counted using a microscope (Nikon, Melville, NY) by two independent investigators in a blinded manner.

Quantification of MCP-1 Protein by ELISA

Quiescent WT and CAST-Tg BMDMs ($1X10^6$/ml) were incubated in a 12 well plate with either vehicle or AngII (100-1,000 nM) for 12 or 24 h. Culture media from BMDMs were collected and centrifuged at 13,000 rpm for 5 minutes. Supernatants were stored at -80°C until assay. Accumulation of MCP-1 protein in media was measured with a mouse MCP-1 ELISA kit (R & D System; catalog No: MJE00) and normalized to cellular protein.

Thioglycollate Elicitation of Peritoneal Macrophages

To elicit peritoneal macrophages, mice were injected intraperitoneally with thioglycollate broth (1 ml; 3% wt/vol). Seventy two hours after thioglycollate injection, mice were sedated and peritoneal macrophages were harvested as described earlier.[18]

mRNA Abundance

Total RNA was extracted from thioglycollate elicitated MPMs, and spleen using the RNeasy Mini Kit (Qiagen; catalog No: 74104) and SV Total RNA Isolation System (Promega; catalog No: Z3100) respectively. RNA (100 ng) was reverse transcribed using the iScript cDNA synthesis kit (Cat #170-8891; Bio-Rad, Hercules, CA). 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. The primers used are detailed in supplemental Table 5.

Western Blot Analyses

Cell or 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: calpain-1 domain IV (Abcam, catalog No: ab39170), calpain-2 (Abcam, catalog No: ab39165), calpastatin (Cell Signaling, catalog No. 4146), α-spectrin (Millipore, catalog No: MAB1622), ABCA1 (Abcam, catalog No: ab7360), ABCG1 (Novus Biologicals, catalog No: 400-132), CD36 (Rat anti-mouse CD36 sera from Dr. Fredrick De Beer's lab, University of Kentucky[19] , SRA-1 (Guinea pig anti mouse SRA-1 sera from Dr. Alan Daugherty's lab, University of Kentucky [20]), NF-kB phospho P-65 (Ser 536) (Cell Signaling, catalog No:3033P), total P-65 (Cell Signaling, catalog No:8242P), IKBα (Cell Signaling, catalog No:4814P) 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.

Statistical Analyses

Data are represented as mean ± SEM. Statistical analyses were performed using SigmaPlot 12.0 (SYSTAT Software Inc., San Jose, CA, USA). 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. Student's *t* test or Mann-Whitney Rank Sum test was performed as appropriate for two-group comparisons. One or Two way ANOVA with Holm-Sidak post hoc analysis was performed for multiple-group and multiple-manipulation analysis. Values of $P<0.05$ were considered to be statistically significant.

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