Leukocyte Calpain Deficiency Reduces Angiotensin II–Induced Inflammation and Atherosclerosis But Not Abdominal Aortic Aneurysms in Mice

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Objective—Angiotensin II (AngII) infusion profoundly increases activity of calpains, calcium-dependent neutral cysteine proteases, in mice. Pharmacological inhibition of calpains attenuates AngII-induced aortic medial macrophage accumulation, atherosclerosis, and abdominal aortic aneurysm in mice. However, the precise functional contribution of leukocyte-derived calpains in AngII-induced vascular pathologies has not been determined. The purpose of this study was to determine whether calpains expressed in bone marrow (BM)–derived cells contribute to AngII-induced atherosclerosis and aortic aneurysms in hypercholesterolemic mice.

Approach and Results—To study whether leukocyte calpains contributed to AngII-induced aortic pathologies, irradiated male low-density lipoprotein receptor−/− mice were repopulated with BM-derived cells that were either wild-type or overexpressed calpastatin, the endogenous inhibitor of calpains. Mice were fed a fat-enriched diet and infused with AngII (1000 ng/kg per minute) for 4 weeks. Overexpression of calpastatin in BM-derived cells significantly attenuated AngII-induced atherosclerotic lesion formation in aortic arches, but had no effect on aeurysm formation. Using either BM-derived cells from calpain-1-deficient mice or mice with leukocyte-specific calpain-2 deficiency generated using cre-loxP recombination technology, further studies demonstrated that independent deficiency of either calpain-1 or -2 in leukocytes modestly attenuated AngII-induced atherosclerosis. Calpastatin overexpression significantly attenuated AngII-induced inflammatory responses in macrophages and spleen. Furthermore, calpain inhibition suppressed migration and adhesion of macrophages to endothelial cells in vitro. Calpain inhibition also significantly decreased hypercholesterolemia-induced atherosclerosis in the absence of AngII.

Conclusions—The present study demonstrates a pivotal role for BM-derived calpains in mediating AngII-induced atherosclerosis by influencing macrophage function. (Arterioscler Thromb Vasc Biol. 2016;36:835-845. DOI: 10.1161/ATVBAHA.116.307285.)

Key Words: angiotensin II • atherosclerosis • calpain • inflammation • macrophages

Angiotensin II (AngII), a major bioactive peptide of the renin–angiotensin system, is a critical mediator of aortic diseases, including atherosclerosis and abdominal aortic aneurysms (AAA).1,2 Chronic infusion of AngII into hypercholesterolemic mice promotes atherosclerosis and leads to the development of AAs.2 AngII-induced atherosclerosis is characterized by an intimal macrophage infiltration that becomes engorged with lipids, whereas AngII-induced AAs are characterized by small focal regions of macrophage accumulation in the aortic media.3 Systemic deficiency of AngII type 1α receptors completely ablates development of AngII-induced atherosclerosis and AAAs in mice.4 However, identities of key regulators and underlying mechanisms for development of these vascular pathologies remain undefined.

Calpains are calcium-dependent intracellular cysteine proteases that tightly regulate their substrate proteins through limited proteolysis.5 The 2 major isoforms, calpain-1 and -2, are expressed ubiquitously, whereas the other isoforms (eg, -3, -9) are tissue-specific.5 Activated calpain by calcium causes damage to cells by selectively degrading intracellular proteins, including signaling proteins (eg, cyclin-dependent kinase, protein kinase C),6,7 cytoskeletal proteins (eg, talin, spectrin),8,9...
and transcription factors (eg, c-Jun, IκB).

Calpains play a critical role in cellular apoptosis through the activation of both caspase-dependent and caspase-independent pathways. Calpains are also involved in acute inflammatory processes via the activation of nuclear factor kappa B (NF-κB). Since calcium-induced calpain activation is an irreversible reaction, calpains are tightly regulated by calpastatin (CAST), which is an endogenous inhibitor that binds strongly to calpains. CAST contains 4 tandem repeats of a calpain-inhibitory domain, and each CAST molecule is capable of inhibiting >1 calpain molecule. Calpains have been implicated to play a critical deleterious role in endothelial dysfunction, hypertension, and fibrosis. Previously, we demonstrated that AngII infusion significantly increased calpain activity and activity in AAA and atherosclerotic lesions by Western blot, an activity assay, and immunohistochemistry. To confirm that the CAST overexpression suppressed calpain activity, we examined the breakdown product of spectrin, a major well-known substrate of calpain. Western blot analyses of cell lysates showed a modest ~2-fold increase in the abundance of CAST protein in BM (Figure 1D) and peritoneal macrophages (Figure 1E) of CAST-Tg mice compared with wild-type (WT) mice. This data confirms the transgenic overexpression of CAST in leukocytes.

To examine the role of leukocyte-derived calpains in AngII-induced aortic pathologies, we confirmed by CAST genotyping of DNA from BM-derived cells of recipient mice after termination (Figure IB in the online-only Data Supplement). Mice were fed a saturated fat–enriched diet and infused with saline or AngII for 4 weeks. To confirm that the CAST overexpression suppressed calpain activity, we examined the breakdown product of spectrin, a major well-known substrate of calpain. Western analyses using protein lysates from macrophages harvested from CAST WT and Tg mice further confirmed increased calpain activity only in WT and not in Tg macrophages as demonstrated by an increased breakdown product of spectrin (Figure 1F). This data clearly suggests a strong inhibition of calpain activity in macrophages by CAST. CAST overexpression in BM-derived cells resulted in a significant decrease (52%; P<0.05) in AngII-induced atherosclerotic lesion areas in aortic arches (WT 20±1.8% versus Tg 9.5±1.0%; Figure 1G and 1H).

To elucidate a functional role for leukocyte-derived calpains in AngII-induced atherosclerosis and AAAs, we repopulated irradiated male low-density lipoprotein (LDL) receptor−/− mice with bone marrow (BM)-derived cells that overexpress CAST, the endogenous inhibitor of calpains. These studies demonstrated that calpain inhibition in leukocytes resulted in decreased atherosclerosis, but not AAAs. Furthermore, using calpain-1 or leukocyte-specific calpain-2-deficient mice, we demonstrated that deficiency of either calpain-1 or -2 in leukocytes modestly, but significantly, attenuated AngII-induced atherosclerosis. Additionally, calpain inhibition significantly modulated inflammatory, migratory, and adhesive properties of macrophages.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Calpains Are Present in AngII-Induced Atherosclerosis
We demonstrated previously that calpains are activated and increased in aortic atherosclerosis and aneurysmal tissue formed during AngII infusion. In this study, we determined the distribution of calpain-1 and -2 in AngII-induced atherosclerosis by immunostaining. Calpain-1 and -2 (Figure 1A and 1B) immunostaining was most pronounced in regions containing macrophages (Figure 1C). Diffused immunostaining was also observed in the aortic media and adventitia.

CAST Overexpression in BM-Derived Cells Decreased AngII-Induced Atherosclerosis
To investigate the role of leukocytic calpains in AngII-induced vascular pathologies, we used the CAST overexpressing transgenic mice to inhibit activities of both calpain-1 and -2. We confirmed the presence of CAST transgene (CAST-Tg) by polymerase chain reaction (PCR) using DNA isolated from BM-derived cells (Figure 1A in the online-only Data Supplement). Western blot analyses of cell lysates showed a modest ~2-fold increase in the abundance of CAST protein in BM (Figure 1D) and peritoneal macrophages (Figure 1E) of CAST-Tg mice compared with wild-type (WT) mice. This data confirms the transgenic overexpression of CAST in leukocytes.

To examine the role of leukocyte-derived calpains in AngII-induced aortic pathologies, irradiated LDL receptor−/− mice were repopulated with BM-derived cells from either WT or CAST-Tg mice. Successful repopulation of donor cells was confirmed by CAST genotyping of DNA from BM-derived cells of recipient mice after termination (Figure IB in the online-only Data Supplement). Mice were fed a saturated fat–enriched diet and infused with saline or AngII for 4 weeks.
in BM-derived cells had no effect on AngII-induced AAA formation (Figure 1M) and aortic rupture (Figure II in the online-only Data Supplement).

**Calpain-1 or -2 Deficiency in Myeloid Cells**

**Modestly Reduced AngII-Induced Atherosclerosis**

To determine the calpain isoform responsible for attenuating atherosclerosis, irradiated male LDL receptor−/− mice were repopulated with BM-derived cells that were either calpain-1+/− or calpain-1−/−. Calpain-1 genotype was confirmed by PCR (Figure IIIA in the online-only Data Supplement). Successful repopulation of donor cells was confirmed by calpain-1 genotyping of DNA from BM-derived cells of recipient mice after termination (Figure IIIB in the online-only Data Supplement). Since calpain-2-deficient mice are embryonically lethal,22 leukocyte-specific calpain-2-deficient LDL receptor−/− mice were generated using calpain-2 floxed (f/f) and LysM Cre transgenic mice. Both calpain-2f/f and LysM Cre transgenic mice were bred to an LDL receptor−/− background. Female calpain-2 f/f mice were bred with male LysM 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 IV in the online-only Data Supplement). Western blot
analyses confirmed depletion of calpain-1 (Figure 2A) and
calpain-2 (Figure 2G) in leukocytes.
Mice were fed a saturated fat–enriched diet and infused
with AngII for 28 days. Calpain-1 or -2 deficiency in myeloid
cells had no effect on body weight, total plasma cholesterol
concentrations, or cell counts (Table II and III in the online-only
Data Supplement). Calpain-1 or -2 deficiency resulted in a
modest, but significant decrease (33% and 31%; \( P<0.05 \)) in
AngII-induced atherosclerosis in aortic arches (Calpain-1:
WT 15±1.2% versus knockout 10±0.96%; Figure 2B and 2C;
Calpain-2 f/f: LysM Cre 0/0 −16±1.4% versus +/0 11±1.2%;
Figure 2H and 2I). Examples of Oil Red O–stained ex vivo
images of mouse aortas representing atherosclerotic lesions
nearest the mean of each group are shown in the Figure
2D, 2E, 2J, and 2K. Similar to CAST-Tg overexpression (Figure
1N; Figure II in the online-only Data Supplement), calpain-1 or

![Figure 2](http://atvb.ahajournals.org/Downloaded from http://atvb.ahajournals.org/)

**Figure 2.** Calpain-1 or -2 deficiency in myeloid cells significantly attenuated angiotensin II (AngII)-induced atherosclerosis. **A,** Calpain-1 protein in mouse peritoneal macrophages (MPMs) from calpain-1+/+ and calpain-1−/− mice. Atherosclerotic lesion area was measured on the intimal surface of aortic arch (**B** and **C**) from LDL receptor−/− mice repopulated with calpain-1+/+ or calpain-1−/− BMs (n=16 each). **D** and **E,** Photographs of Oil Red O–stained mouse aortas representing atherosclerotic lesions nearest the mean of each group. **F,** Maximal external width of abdominal aortas. **G,** Calpain-2 protein were detected in cell and tissue lysates from calpain-2 f/f LysM Cre0/0 and +/0 mice. Atherosclerotic lesion area was measured on the intimal surface of aortic arch (**H** and **I**) from AngII-infused calpain-2 f/f mice that were either Cre 0/0 or +/0 (n=13). **J** and **K,** Photographs of Oil Red O–stained mouse aortas representing atherosclerotic lesions nearest the mean of each group. **L,** Maximal external width of abdominal aortas. White (+/+/Cre 0/0) and gray circles (−/−/Cre +/0) represent individual mice, diamonds represent means, and bars are SEMs. * denotes \( P<0.05 \) when comparing +/+/Cre 0/0 vs −/−/Cre +/0 mice (Mann–Whitney rank-sum test).
-2 deficiency in myeloid cells had no effect on AngII-induced AAA formation (Figure 2F and 2L) and aortic rupture (Figure V in the online-only Data Supplement).

**CAST Overexpression Attenuated Macrophage Accumulation**

Since macrophage accumulation is one of the earliest events occurring in the development of atherosclerosis, we examined the contribution of leukocytic calpains on macrophage accumulation in AngII-induced atherosclerosis. Histology and immunostaining of AngII-induced atherosclerotic lesions of WT and CAST-Tg mice using H&E stain and an anti-CD68 antibody revealed accumulation of CD68+ macrophages in atherosclerotic lesions (Figure 3A–3D). Compared with WT, CAST Tg overexpression in BM-derived cells had less macrophage recruitment to the smaller atherosclerotic lesions.

In addition to lesional macrophage accumulation, further histological and immunostaining analyses of aortic arches from WT and CAST-Tg mice infused with AngII revealed occurrence of focal elastin layer disruption (Figure VIA, VIB, VII, VIJ in the online-only Data Supplement) associated with the accumulation of CD68+ macrophages (Figure VIE and VIF in the online-only Data Supplement). However, CAST overexpression attenuated AngII-induced medial elastin layer disruption (Figure VIC and VID in the online-only Data Supplement) and medial macrophage accumulation (Figure VIG and VIH in the online-only Data Supplement). The specificity of immunostaining was verified using appropriate negative controls (Figure VII in the online-only Data Supplement).

**CAST Overexpression Had No Effect on Macrophage Lipid Uptake**

To define whether calpains influence macrophage lipid uptake as a mechanism in mediating atherosclerosis, we examined the effect of CAST overexpression on mRNA and protein abundance of ATP-binding cassette (ABC) transporters and other atherosclerosis-associated genes in macrophages. WT and CAST-Tg BM-derived macrophages (BMDMs) were incubated with either vehicle or acetylated LDL (AcLDL; 25 μg/mL) for 24 or 48 hours. Cells were lysed to harvest either mRNA or protein.

mRNA abundance of ABCA1, G1, SRA-1, CD36, ACAT, and LXRα were examined by quantitative reverse transcriptase...
PCR. The primers used are detailed in Table V in the online-only Data Supplement. AcLDL incubation significantly upregulated mRNA abundance of ABCA1 (24 and 48 hours), ABCG1 (48 hours), LXRα (24 and 48 hours), and downregulated SRA-1 (48 hours; Figure VIII in the online-only Data Supplement). These alterations in the mRNA abundance of ABCA1, ABCG1, SRA-1, LCAT, and LXRα were not influenced by CAST overexpression (Figure VIII in the online-only Data Supplement). Interestingly, CAST overexpression only showed a significant upregulation of CD36 mRNA after 24-hour AcLDL incubation compared with WT-BMDMs. At the protein level, compared with WT-BMDMs, CAST overexpression showed an increase in ABCA1 protein on AcLDL incubation. CAST overexpression had no influence on the abundance of ABCG1, CD36, and SRA-1 protein (Figure IX in the online-only Data Supplement).

Next, we examined the effect of CAST overexpression on macrophage lipid uptake. WT and CAST-Tg BMDMs were incubated with either vehicle or fluorescent Dil-labeled AcLDL (25 μg/mL) for 24 hours. Dil-labeled AcLDL was used to trace the AcLDL uptake. Dil-AcLDL uptake was assessed by fluorescent microscopy (Figure 3E–3I and flow cytometry (Figure 3J and 3K). The cells were washed with ice-cold acid buffer before detection to avoid the contamination of surface sticking AcLDL. Results from both analyses showed that AcLDL uptake was not impaired by calpain inhibition. Therefore, calpain inhibition in macrophages by CAST overexpression had no effect on macrophage lipid uptake.

To further investigate whether AngII infusion had any effect on atherosclerosis-associated genes in macrophages, CAST WT and Tg mice were infused with either saline or AngII. Peritoneal macrophages were elicited using thioglycollate (1 mL; 3% wt/vol) 72 hours prior harvest as described previously.20 mRNA abundance of ABCA1, ABCG1, SRA-1, CD36, and LXRα were examined in peritoneal macrophages. Neither AngII infusion nor CAST overexpression influenced mRNA abundance of these atherosclerosis-associated genes (Figure X in the online-only Data Supplement).

CAST Overexpression Attenuated Macrophage Adhesion and Migration

To define potential mechanisms by which calpain influenced macrophage accumulation in atherosclerotic lesions, we investigated whether calpain inhibition influenced functional properties of macrophages, such as migration and adhesion to endothelial cells (ECs). The adhesion assay was performed on mouse aortic ECs. The EC purity was verified using a biotin-labeled CD31 antibody by immunohistochemistry (Figure XI in the online-only Data Supplement). The ECs were stimulated with AngII (1 μmol/L) overnight. Co-incubation of BMDMs with stimulated ECs showed that AngII significantly promoted macrophage adhesion to ECs. Compared with WT-BMDMs, CAST Tg overexpression significantly suppressed BMDM adhesion to ECs both under basal and under AngII conditions (Figure 4A–4E).

In addition, the effect of CAST overexpression on macrophage migration toward monocyte chemoattractant protein-1 (MCP-1) was examined by transwell migration assay. MCP-1 strongly stimulated migration of macrophages from WT mice compared with vehicle control, whereas overexpression of CAST significantly reduced MCP-1-induced macrophage migration (Figure 4F–4I). Therefore, calpain inhibition in macrophages by CAST overexpression decreased the migration and adhesion abilities of macrophages.

Figure 4. Calpastatin (CAST) overexpression significantly reduced macrophage adhesion and migration. A–D, Calcein-labeled wild-type (WT) and CAST-transgene (Tg) bone marrow–derived macrophages (BMDMs) were added to a mouse aortic endothelial cell monolayer for 60 minutes. Adhered cells were counted from 10 fields at the power of 100× magnification using a fluorescence microscope and quantified (E; n=4). F–I, WT and CAST-Tg BMDMs were seeded on transwell filters, and lower chambers were filled with media containing either vehicle or monocyte chemoattractant protein-1 (MCP-1; 100 μg/mL). Cells that migrated through the membrane stained with H&E and were counted from 9 fields at the power of 200× magnification (J; n=4). Horizontal lines represent significance of P<0.05. * and # denote P<0.05 when comparing AngII vs saline and WT vs Tg, respectively (2-way analysis of variance [ANOVA] with Holm–Sidak post hoc analysis).
CAST Overexpression Attenuated AngII-Induced Inflammation

To further investigate the role of calpain on AngII-induced inflammation, WT and CAST-Tg BMDMs were incubated with either vehicle or AngII. AngII incubation failed to show any effect on secretion or induction of MCP-1 (Figure XIIA and XIIB in the online-only Data Supplement) or other inflammatory genes (eg, interleukin [IL]-6) expression from cultured BMDMs at 12 or 24 hours (data not shown). However, BMDMs incubated with positive-control lipopolysaccharide showed a strong induction of MCP-1 (Figure XIIA and XIIB in the online-only Data Supplement). Since we failed to observe any response of AngII in cultured BMDMs, next we used an in vivo approach in which WT and CAST-Tg mice were infused with either saline or AngII for 7 days. Peritoneal macrophages were elicited using thioglycollate (1 mL; 3% wt/vol) 72 hours prior harvest as described previously.20 mRNA or protein were extracted from the mouse peritoneal macrophages and subjected to quantitative PCR and Western blot analyses. mRNA abundance of MCP-1, inflammatory cytokines (IL-6, IL-10), and NF-kB-related inhibitor of kappa B kinases (IKKa, β, and ε) were examined in peritoneal macrophages.

Figure 5. Calpastatin (CAST) overexpression significantly reduced angiotensin II (AngII)–induced monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-6 and nuclear factor kappa B (NF-kB)–dependent inflammatory genes in macrophages. A, mRNA abundance of MCP-1, IL-6, IL-10, and inhibitor of kappa B kinase genes in mouse peritoneal macrophage (MPMs) from saline and AngII-infused low-density lipoprotein (LDL) receptor−/− mice transplanted with WT or CAST-transgene (Tg) bone marrow (BMs) were analyzed by quantitative polymerase chain reaction (qPCR; n=4–6). B, Western blot analyses of phospho and total P65, and IkBα protein in MPMs from saline and AngII-infused LDL receptor−/− mice transplanted with wild-type (WT) or CAST-Tg BMs (n=4). Values are represented as mean±SEM. * and # denote P<0.05 when comparing AngII vs saline and WT vs Tg, respectively (2-way ANOVA with Holm–Sidak post hoc analysis).
and spleen. The primers used are detailed in Table V in the online-only Data Supplement. AngII infusion significantly increased mRNA abundance of MCP-1, IL-6, and IKKs and had no effect on IL-10 in macrophages (Figure 5A) and spleen (Figure XIII in the online-only Data Supplement). In contrast, CAST overexpression significantly reduced AngII-induced MCP-1, IL-6, and IKKs gene expression.

IKK-dependent NF-kB activation has been implicated in the development of atherosclerosis. Activated NF-kB is also shown to induce inflammatory cytokines, such as IL-6 and tumor necrosis factor-α. NF-kB is sequestered in the cytosol, and its translocation to the nucleus is prevented by the inhibitor of NF-kB translocation (IkB). IkB was also shown to be a target of calpain in selected cell types, including macrophages. To investigate the possibility that calpain activation mediates AngII-induced NF-kB activation and IkB degradation, phosphorylation of NF-kB subunit P65 and IkB protein was examined by Western blot. AngII infusion significantly reduced IkB protein and significantly increased phosphorylation of P65 in WT mouse peritoneal macrophages (Figure 5B). In contrast, CAST overexpression significantly prevented AngII-induced IkB protein degradation and P65 phosphorylation in macrophages (Figure 5B).

CAST Overexpression in BM-derived Cells Decreased Hypercholesterolemia-Induced Atherosclerosis

To understand the role of endogenous calpains in leukocytes on atherosclerosis in the absence of exogenous stimuli, AngII, we examined the role of leukocytic calpains in the development of hypercholesterolemia-induced atherosclerosis in the absence of AngII. Since studies highlighted in Figures 1 and 2 showed that deficiency of calpain-1 or -2 in leukocytes resulted in a modest decrease (33% and 31%; P<0.05) in AngII-induced atherosclerosis (Figure 2), whereas inhibition of both calpain-1 and -2 by overexpression of its endogenous inhibitor, CAST, significantly decreased (52%; P<0.05) AngII-induced atherosclerotic lesion areas in aortic arches (Figure 1H and 1I). Based on these observations, we examined the effect of leukocytic calpains on hypercholesterolemia-induced atherosclerosis using CAST overexpression Tg mice instead of calpain-1 or -2 isoform–specific deficient mice.

To examine the role of leukocyte-derived calpains on hypercholesterolemia-induced atherosclerosis, irradiated LDL receptor−− mice were repopulated with BM-derived cells from either WT or CAST-Tg mice. Successful repopulation of donor cells was confirmed by CAST genotyping of DNA from BM-derived cells of recipient mice after termination (Figure IC in the online-only Data Supplement). Mice were fed a saturated fat–enriched diet for 12 weeks. CAST overexpression had no effect on body weight gain, plasma total cholesterol concentrations, or blood cell counts (Table IV in the online-only Data Supplement). CAST overexpression suppressed hypercholesterolemia-induced calpain activity, as demonstrated by decreased breakdown of its known substrate, spectrin, in macrophages harvested from WT and Tg mice fed with high-fat diet (Figure 6A). This data clearly suggests a strong inhibition of calpain activity in macrophages by CAST. CAST overexpression in BM-derived cells resulted in a significant decrease (58%; P<0.05) in hypercholesterolemia-induced atherosclerotic lesion areas in aortic arches (WT 4.2±0.3% versus Tg 1.8±0.3%; Figure 6B and 6C). Examples of ex vivo images of Oil Red O–stained aortas representing atherosclerotic lesions nearest the mean of each group are shown in the Figure 6D and 6E.

Discussion

Activated calpain has been implicated in AngII-induced aortic pathologies, such as atherosclerosis and AAAs. However, the contribution of leukocytic calpains on the development of AngII-induced vascular pathologies is not known. In the present study, we generated 3 different mice models: (1) chimeric LDL receptor−− mice that overexpress CAST, an endogenous inhibitor of calpains, in BM-derived cells, (2) chimeric LDL receptor−− mice with either calpain-1+/− or calpain-1−/− BM cells, and (3) LDL receptor−− mice with

Figure 6. Calpastatin (CAST) overexpression significantly reduced hypercholesterolemia-induced atherosclerosis. A, Spectrin full-length protein and breakdown product was detected in macrophages from high-fat diet–fed low-density lipoprotein (LDL) receptor−− mice transplanted with wild-type (WT) or CAST-transgene (Tg) bone marrow (BMs; n=4). Atherosclerotic lesion area was measured on the intimal surface of the aortic arch (B and C; n=14). D and E, Photographs of Oil Red O–stained mouse aortas representing atherosclerotic lesions nearest the mean of each group. White (WT) and gray circles (Tg) represent individual mice, diamonds represent means, and bars are SEMs. * denotes P<0.05 when comparing WT vs Tg mice (Mann–Whitney rank-sum test).
leukocyte-specific calpain-2 deficiency. Using these 3 different calpain-deficient mice models, we examined the role of leukocyte calpains in AngII-induced atherosclerosis and AAAs. Here, we demonstrated that combined inhibition of both calpain-1 and -2 activities significantly reduced AngII-induced atherosclerosis, without influencing AAA formation. The beneficial effect of calpain inhibition on AngII-induced atherosclerosis was associated with the reduction of macrophage accumulation and inflammation, which attributed to blunted functional properties of macrophages, including migration and adhesion. Furthermore, we demonstrated that calpain inhibition also suppressed hypercholesterolemia-induced atherosclerosis in mice.

Overexpression of CAST or calpain-1 or -2 deficiency in leukocytes did not have any effects on AngII-induced blood pressure elevation. Consistent with the current observation, our earlier study of calpain inhibition using BDA-410 or calpain-1 deficiency also showed no effect on AngII-induced increased blood pressure.20,21 This result is in agreement with an earlier study by Letaverner et al, in which overexpression of calpastatin attenuated AngII-induced cardiac hypertrophy without affecting blood pressure.19 Further, development of AngII-induced AAA and atherosclerosis was also shown to be independent of increases in blood pressure.29

Calpain inhibition in BM-derived cells by CAST overexpression significantly reduced AngII-induced atherosclerosis. In agreement, depletion of AngII type 1a receptors in BM-derived cells, not endothelial or smooth muscle cells, partially reduced AngII-accelerated atherosclerosis. Our recent study clearly demonstrated that AngII promoted calpain activity in macrophages, made evident by increased breakdown product of spectrin, a well-known substrate of calpain. Furthermore, calpain inhibition in BM-derived cells reduced both AngII-induced and hypercholesterolemia-induced atherosclerosis without affecting plasma cholesterol concentrations. In addition, calpain inhibition had no influence on macrophage lipid (Ac-LDL) uptake under in vitro conditions. On lipid loading, CAST overexpression showed an increase in the protein abundance of ABCA1, whereas it had no influence on mRNA and protein abundance of ABCG1 and other atherosclerosis-associated genes (eg, SR-A1, CD36) in macrophages. Macrophage ABCA1 transporter deficiency is shown to promote atherosclerosis in mice by increasing plaque inflammation and suppressing cholesterol efflux pathways.31,32 Pharmacological inhibition of calpain or mutation of calpain-binding PEST (proline, glutamic acid, serine, and threonine) sequence in ABCA1 showed that calpain degrades ABCA1 by proteolysis.33-35 The observed beneficial effect of CAST Tg overexpression on AngII/hypercholesterolemia-induced atherosclerosis may be because of improved cholesterol efflux from macrophages rather than lipid uptake. These current findings suggest that calpain activation promotes atherosclerosis by influencing functional properties of macrophages, such as migration, adhesion, and subsequent inflammation without influencing macrophage lipid uptake. However, further studies are warranted to understand whether calpain has any role on ABCA1-mediated macrophage cholesterol efflux and reverse cholesterol transport in promoting atherosclerosis. Interestingly, the combined inhibition of calpain-1 and -2 by CAST overexpression showed a strong reduction in atherosclerosis over independent depletion, suggesting a synergistic role of calpain-1 and -2 in reducing atherosclerosis. However, calpain inhibition in leukocytes had no influence on AngII-induced AAA formation. In agreement, previous studies showed that BM transplantation using AngII type 1a receptor-deficient mice had no effect on AngII-induced AAA development.3 Our earlier studies clearly demonstrated that whole body calpain inhibition attenuated AngII-induced AAAs, suggesting that calpain derived from vessel wall cells are involved in AAA formation.20,21

Calpain inhibition reduced AngII-induced proinflammatory gene expression, such as IKKs, MCP-1, and IL-6, in macrophages. Furthermore, calpain inhibition in macrophages impaired migration and adhesion properties, which may have contributed to decreased AngII-induced atherosclerosis.36,37 In addition, calpain inhibition suppressed AngII-induced NF-kB activation (P65 phosphorylation) and prevented AngII-induced IkB degradation in macrophages. IKK-dependent NF-kB activation has been implicated in the development of atherosclerosis.37 Recently, LDL receptor−/− mice with macrophage-specific IKKβ deficiency had less hypercholesterolemia-induced atherosclerosis. NF-kB translocation inhibitor, Ikβ, was also shown to be a target of calpain in selected cell types, including macrophages.25-28 In support, CAST deficiency in intestinal macrophages also induced NF-kB translocation to the nucleus. Further, depletion of calpain-4, the common small subunit of calpain-1 and -2 in cardiomyocytes, resulted in suppressed AngII-derived NF-kB activity followed myocardial infarction in mice.51 NF-kB activation plays an important role in promoting expression of various proinflammatory factors (eg, MCP-1, IL-6), which mediate inflammatory responses.38,39 MCP-1 and IL-6 play important roles in aortic medial macrophage recruitment and inflammation in mice. Mice deficient with chemokine (C-C motif) receptor 2, the cognate receptor of MCP-1, have attenuated AngII-induced atherosclerosis and AAAs.40 In addition, in the elastase-induced AAA model, MCP-1-deficient mice displayed a strong reduction in macrophage infiltration.41 Further, MCP-1-deficient mice also showed significant reduction in hypercholesterolemia-induced atherosclerosis.42 Mice deficient in IL-6 had reduced AngII-induced aortic dissection and inflammation partially by suppressing MCP-1-mediated macrophage recruitment.43 In the present study, the suppressed NF-kB activation may result in decreased downstream events, such as induction of inflammatory cytokines, MCP-1, and IL-6. This may contribute to decreased migration, adhesion, and inflammation of CAST-overexpressing macrophages. In line, the expression of AngII-induced MCP-1, IL-6, and NF-kB P65 phosphorylation were significantly decreased in CAST-overexpressing macrophages. This reduction in macrophage inflammation, in addition to, impaired macrophage migration and adhesion on CAST overexpression may well correlate with the observed reduction of macrophage infiltration into AngII-induced atherosclerotic lesions in the aortic arch.

Since CAST overexpression had no effect on changes in plasma cholesterol concentrations and macrophage lipid uptake, our results suggest that an inhibitory effect on macrophage inflammation, migration, and accumulation may be the key mechanism by which calpain inhibition attenuates
AngII-induced atherosclerosis. Thus, it is postulated that AngII-induced calpain activation promotes NF-κB activation, resulting in the induction of proinflammatory cytokines—MCP-1 and IL-6, which in turn stimulates macrophage migration and adhesion, thereby causing atherosclerosis. However, future studies are required to further understand the mechanisms through which calpain regulates functional properties of macrophages under inflammatory conditions.

In summary, we demonstrated that inhibition of leukocyte-derived calpains resulted in decreased atherosclerosis, which was associated with reduced macrophage inflammation, migration, and adhesion. These results suggest that inhibition of calpain activity may offer a new therapeutic target to reduce atherosclerosis.

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Disclosures

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References

Significance

Pharmacological inhibition of calcium-dependent cysteine protease, calpains, decreases angiotensin II–induced atherosclerosis, abdominal aortic aneurysms, and medial macrophage accumulation in low-density lipoprotein receptor–/– mice, independent of plasma cholesterol concentrations. This finding suggests that calpain inhibition exerts an antiatherogenic effect mediated by modulation of inflammatory responses in atherosclerotic plaques. Here, we evaluated this hypothesis by investigating the contribution of macrophage-derived calpains to development of angiotensin II–induced aortic pathologies. Using leukocyte-specific calpain-deficient mice, we demonstrated that (1) leukocyte-derived calpains play a critical role in the development of angiotensin II–induced atherosclerosis; (2) both calpain-1 and -2, the major ubiquitous isoforms, play a synergistic role in accelerating atherosclerosis; (3) inhibition of calpain activity in macrophages reduces macrophages migration, adhesion to endothelial cells, and accumulation in atherosclerotic lesions. These results suggest that targeted inhibition of calpain activity may offer a new therapeutic direction to reduce atherosclerosis.
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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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a Indicates equal contribution

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. Calpain-1 -/- mice on a C57BL/6 background were generated originally in the laboratory of Dr. Athar Chishti. Calpain-2 floxed (f/f) mice on a C57BL/6 background were originally generated in the laboratory of Dr. Takaomi Saido. 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'-GTTGCGCTAGGCTCCTTTCTGT-3' and 5'-CCAGACTCGTGAACCCCTT-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'-CTAGGCCACAGAATTGAAAGATCT and 5'-GTAGGTGAAATTCTAGCATCATCC. The resultant product was 324 bp (Supplemental Figure S1). Calpain-1 genotyping used the following primers: 5'-TGCACCTCTAGTGGAGTT-3', 5'-AGAGTGCACGAACACCAGCTT-3', and 5'-TTAAGGGCCAGCTCATCC. The resultant product was 324 bp (Supplemental Figure S1). Calpain-1 genotyping used the following primers: 5'-TGCACCTCTAGTGGAGTT-3', 5'-AGAGTGCACGAACACCAGCTT-3', and 5'-TTAAGGGCCAGCTCATCC. The resultant product was 324 bp (Supplemental Figure S1). Calpain-1 genotyping used the following primers: 5'-TGCACCTCTAGTGGAGTT-3', 5'-AGAGTGCACGAACACCAGCTT-3', and 5'-TTAAGGGCCAGCTCATCC. The resultant product was 324 bp (Supplemental Figure S1). Calpain-2f/f genotyping used the following primers: 5'-ATACTGCTCTGTGATACG-3' and 5'-CTCTGTCAGGCTCATGTCCTCCAGAGGATG-3'. Resultant wild-type, and flox allele bands were 290, and 430 bp, respectively (Supplemental Figure S1). 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 x 10⁶ 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. 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.

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. 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. 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. Bone marrow cells were harvested from the femurs of WT and CAST-Tg mice 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 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 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. 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 (1x10⁶ 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). 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). WT and CAST-Tg BMDMs were seeded (1x10⁶ 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 (1X10⁶/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 Kentucky19, SRA-1 (Guinea pig anti mouse SRA-1 sera from Dr. Alan Daugherty’s lab, University of Kentucky20), NF-kB phospho P-65 (Ser 536) (Cell Signaling, catalog No:3033P), total P-65 (Cell Signaling, catalog No:8242P), IκBα (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.
REFERENCES


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19. de Villiers WJ, Cai L, Webb NR, de Beer MC, van Der Westhuyzen DR, de Beer FC. Cd36 does not play a direct role in hdl or idl metabolism. *J Lipid Res*. 2001;42:1231-1238

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\textsuperscript{a} Indicates equal contribution

Running Title: Leukocyte Calpain Deficiency Reduces Atherosclerosis

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Table I. Effects of calpastatin overexpression in BM-derived cells in male LDL receptor -/- mice infused with saline or AngII

<table>
<thead>
<tr>
<th>Groups</th>
<th>CAST WT</th>
<th>CAST Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infusion</td>
<td>Saline</td>
<td>AngII</td>
</tr>
<tr>
<td>N</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>27.7 ± 1.6</td>
<td>25.0 ± 1.5</td>
</tr>
<tr>
<td>Plasma Cholesterol (mg/dL)</td>
<td>1091 ± 89</td>
<td>1613 ± 33#</td>
</tr>
<tr>
<td>Systolic BP Pre-Infusion (mmHg)</td>
<td>159 ± 3</td>
<td>150 ± 3</td>
</tr>
<tr>
<td>Systolic BP Post-Infusion (mmHg)</td>
<td>158 ± 3</td>
<td>193 ± 5*</td>
</tr>
<tr>
<td>WBC (10^3/µl)</td>
<td>4.9 ± 0.9</td>
<td>5.0 ± 0.3</td>
</tr>
<tr>
<td>RBC(10^6/µl)</td>
<td>8.8 ± 0.5</td>
<td>11.7 ± 0.1</td>
</tr>
<tr>
<td>Platelets(10^3/µl)</td>
<td>617 ± 29</td>
<td>514 ± 19</td>
</tr>
</tbody>
</table>

Values are represented as means ± SEMs. Body weights, plasma cholesterol concentrations, and blood cell counts were determined at termination. Systolic blood pressure was measured prior to (week 0) and during AngII infusion (week 4). Two way repeated measures ANOVA was used to analyze systolic blood pressures. * Denotes P<0.05 systolic BP post-infusion vs pre-infusion, by two-way repeated measures ANOVA. \# denotes P<0.05 for plasma cholesterol CAST WT AngII vs CAST WT Saline, by two-way ANOVA. There were no significant differences between the CAST genotypes for body weight, plasma cholesterol, systolic BP and blood cell counts.
Table II. Effects of calpain-1 deficiency in BM-derived cells in male LDL receptor -/- mice infused with AngII

<table>
<thead>
<tr>
<th>Groups</th>
<th>Calpain-1 +/-</th>
<th>Calpain-1 -/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>25.9 ± 1.6</td>
<td>26.6 ± 1.6</td>
</tr>
<tr>
<td>Plasma Cholesterol Concentrations (mg/dL)</td>
<td>1410 ± 68</td>
<td>1344 ± 36</td>
</tr>
<tr>
<td>Systolic BP Pre-infusion (mmHg)</td>
<td>142 ± 2</td>
<td>141 ± 3</td>
</tr>
<tr>
<td>Systolic BP Post-infusion (mmHg)</td>
<td>171 ± 4*</td>
<td>170 ± 4*</td>
</tr>
<tr>
<td>WBC (10^3/µl)</td>
<td>4.0 ± 0.5</td>
<td>3.4 ± 0.6</td>
</tr>
<tr>
<td>RBC(10^6/µl)</td>
<td>10.0 ± 0.6</td>
<td>10.1 ± 0.2</td>
</tr>
<tr>
<td>Platelets(10^3/µl)</td>
<td>592 ± 54</td>
<td>667 ± 36</td>
</tr>
</tbody>
</table>

Values are represented as means ± SEMs. Body weights, plasma cholesterol concentrations, and blood cell counts were measured at termination. One way repeated measures ANOVA was used to analyze systolic blood pressures. * Denotes P<0.001 for systolic BP post-infusion vs pre-infusion, by one-way repeated measures ANOVA. There were no significant differences between calpain-1 genotypes for body weight, plasma cholesterol, systolic BP and blood cell counts.
Table III. Effects of calpain-2 deficiency in leukocytes in male LDL receptor -/- mice infused with AngII

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cre 0/0</th>
<th>Cre +/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>27.4 ± 0.9</td>
<td>27.3 ± 0.5</td>
</tr>
<tr>
<td>Plasma Cholesterol Concentrations (mg/dL)</td>
<td>1820 ± 96</td>
<td>1825 ± 68</td>
</tr>
<tr>
<td>Systolic BP Pre-infusion (mmHg)</td>
<td>156 ± 6</td>
<td>157 ± 6</td>
</tr>
<tr>
<td>Systolic BP Post-infusion (mmHg)</td>
<td>191 ± 4*</td>
<td>195 ± 6*</td>
</tr>
<tr>
<td>WBC (10³/µl)</td>
<td>5.6 ± 0.6</td>
<td>6.6 ± 0.6</td>
</tr>
<tr>
<td>RBC(10⁶/µl)</td>
<td>10 ± 0.3</td>
<td>9.9 ± 0.4</td>
</tr>
<tr>
<td>Platelets(10³/µl)</td>
<td>650 ± 36</td>
<td>693 ± 25</td>
</tr>
</tbody>
</table>

Values are represented as means ± SEMs. Body weights, plasma cholesterol concentrations, and blood cell counts were determined at termination. One way repeated measures ANOVA was used to analyze systolic blood pressures. * Denotes $P<0.001$ systolic for BP post-infusion vs pre-infusion, by one-way repeated measures ANOVA. There were no significant differences between calpain-2 LsyM-Cre genotypes for body weight, plasma cholesterol, systolic BP and blood cell counts.
Table IV. Effects of calpastatin overexpression in BM-derived cells in male LDL receptor -/- mice fed with hypercholesterolemic diet

<table>
<thead>
<tr>
<th>Groups</th>
<th>CAST WT</th>
<th>CAST Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>33.0 ± 1.0</td>
<td>33.1 ± 1.0</td>
</tr>
<tr>
<td>Plasma Cholesterol Concentrations (mg/dL)</td>
<td>1375 ± 93</td>
<td>1511 ± 75</td>
</tr>
<tr>
<td>Systolic BP Pre HCD (mmHg)</td>
<td>155 ± 4</td>
<td>148 ± 3</td>
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<tr>
<td>Systolic BP Post HCD (mmHg)</td>
<td>146 ± 4</td>
<td>138 ± 3</td>
</tr>
<tr>
<td>WBC (10^3/µl)</td>
<td>6.3 ± 0.8</td>
<td>5.4 ± 0.3</td>
</tr>
<tr>
<td>RBC(10^6/µl)</td>
<td>8.1 ± 0.7</td>
<td>9.0 ± 0.2</td>
</tr>
<tr>
<td>Platelets(10^3/µl)</td>
<td>695 ± 49</td>
<td>638 ± 48</td>
</tr>
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</table>

Values are represented as means ± SEMs. Body weights, plasma cholesterol concentrations, and blood cell counts were determined at termination. One way repeated measures ANOVA was used to analyze systolic blood pressures. There were no significant differences between the CAST genotypes for body weight, plasma cholesterol, systolic BP and blood cell counts.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Product size (bp)</th>
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<tbody>
<tr>
<td>MCP-1</td>
<td>5’-CAGCCAGATGCAGTTAACCAGC 5’-TCTGGACCCATTCTTTG</td>
<td>175</td>
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<tr>
<td>IL-6</td>
<td>5’-GGGAAATCGTGGAATGAGAA 5’-AAGTGACACATCGTTGTTCCATAA</td>
<td>167</td>
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<tr>
<td>IL-10</td>
<td>5’-CCAAGCCCTTATCGGAATGA 5’-TCTCACCACGCAGGAATTTCAA</td>
<td>190</td>
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<tr>
<td>IKKα</td>
<td>5’-GTCAGGACCCTGTTTCTCAAGG 5’-GCTTCTTTTGATGTTACTGAGGCC'</td>
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<tr>
<td>IKKβ</td>
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<td>5’-ACCACTAACTACCTGTGGCAT 5’-CCTCCACTCGGAATAGCTTC</td>
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<tr>
<td>ABCA1</td>
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Supplementary Figure I. Genotyping of experimental mice for the CAST transgene (Tg) by PCR.

A. Genomic DNA from BM-derived cells was isolated and screened by PCR for the CAST-Tg allele. IL-2 was used as an internal control. **B and C.** PCR confirmed CAST-Tg genotype in BM-derived cells harvested from chimeric mice. PCR on BM-derived cells yielded amplicons of 518 and 324 bp for CAST-Tg and IL-2 alleles, respectively. (M= Molecular weight ladder)
Supplementary Figure II. CAST overexpression had no effect on AngII-induced aortic rupture.
Mortality due to AAA rupture in AngII infused LDL receptor-/- mice transplanted with WT or CAST-Tg BMs. White (WT) and gray bar (Tg).
Supplementary Figure III. Genotyping of experimental mice for calpain-1 alleles by PCR.

A. Genomic DNA from BM-derived cells was isolated and screened by PCR for calpain-1 wild type (+/+ ) and null (-/- ) alleles. B. PCR confirmed calpain-1 genotypes in BM-derived cells harvested from chimeric mice. PCR on BM-derived cells yielded amplicons of 615 and 415 bp for calpain-1 +/+ and -/- alleles, respectively. (M= Molecular weight ladder)
Supplementary Figure IV. Genotyping of experimental mice for the calpain-2 floxed allele and Cre Tg by PCR. A. PCR screening strategy to verify the presence of calpain-2 flox/flox. A 430 bp product was generated from calpain-2 f/f mice, while a 290 bp product was obtained from calpain-2 WT mice. Genomic DNA from tail biopsies was isolated and screened by PCR. B. Cre Tg was confirmed by PCR using IL-2 gene as internal control. PCR on genomic DNA yielded amplicons of 182 and 324 bp for Cre Tg and IL-2 alleles, respectively. (M= Molecular weight ladder)
Supplementary Figure V. Calpain-1 or-2 deficiency in myeloid cells had no effect on AngII-induced aortic rupture.  A. Mortality due to AAA rupture in AngII infused LDL receptor-/- mice transplanted with calpain-1 +/+ or -/- BM cells.  B. Mortality due to AAA rupture in AngII-infused calpain-2 f/f mice that were either LysM Cre 0/0 or +/+. White (+/+ / Cre0/0) and gray bar (-/- / Cre+/-).
Supplementary Figure VI. CAST overexpression reduced macrophage accumulation in AngII-infused aortic arch.
Representative aortic arch (200x magnification) from AngII-infused LDL receptor-/- mice transplanted with WT or CAST-Tg BMs stained with H&E (A-D), immunostained for CD68 (E-H) and α-actin (I-L). Arrows indicate positive staining (red).
Supplementary Figure VII. Representative images of negative controls for immunostaining on arch atherosclerotic lesions. Representative aortic arch atherosclerotic lesion sections stained with rat IgG (A), rabbit IgG (B), only secondary antibodies (Rabbit anti-rat or Goat anti-rabbit) and no antibodies. Scale bars correspond to 50 μm (200x magnification).
Supplementary Figure VIII. CAST overexpression had no effect on atherosclerosis-associated genes in lipid-loaded macrophages.

mRNA abundance of ABCA1, ABCG1, SRA-1, CD36, ACAT and LXRα in WT and CAST Tg BMDMs incubated with vehicle (V) or Ac-LDL (Ac; 25 µg/ml) (n=4). Values are represented as mean ± SEM. * denotes P<0.05 when comparing AcLDL vs Vehicle (Two-way ANOVA with Holm-Sidak post hoc analysis).
Supplementary Figure IX. CAST overexpression had no effect on protein abundance of ABC transporters, CD36 and SRA in lipid-loaded macrophages.
Western blot analyses of ABCA1, ABCG1, CD36 and SRA in WT and CAST Tg BMDMs incubated with Ac-LDL (25 µg/ml) (n=4).
Supplementary Figure X. CAST overexpression had no effect on atherosclerosis-associated genes in mouse peritoneal macrophages. mRNA abundance of ABCA1, ABCG1, SRA-1, CD36, ACAT and LXRα in MPMs from saline and AngII infused LDL receptor-/- mice transplanted with WT or CAST-Tg BMs were analyzed by qPCR (n=4-6). Values are represented as mean ± SEM. (Two-way ANOVA with Holm-Sidak post hoc analysis).
Supplementary Figure XI. CD31 staining on mouse aortic endothelial cells. Representative images of aortic endothelial cells immunostained for CD31, SMC $\alpha$-actin, ERTR7 (fibroblasts) and appropriate IgG, secondary antibodies controls. CD31+ cells stain red. Scale bars correspond to 50 μm (200x magnification).
Supplementary Figure XII. AngII had no effect on MCP-1 production in BMDMs.  

A. MCP-1 protein accumulated in cell culture media was measured by ELISA (n=4).  

B. MCP-1 mRNA abundance was analyzed by real-time PCR using 18S as an internal control (n=4).  Values are represented as means ± SEMs. Statistical analyses were performed using one-way ANOVA followed by Holm-Sidak post hoc tests.  * denotes $P<0.05$ when comparing LPS vs vehicle.
Supplementary Figure XIII. CAST overexpression reduced AngII-induced inflammation in spleen. mRNA abundance of MCP-1, IL-6, IL-10, and IKK genes in spleen from saline and AngII infused LDL receptor-/- mice transplanted with WT or CAST-Tg BMs were analyzed by qPCR (n=4-6). Values are represented as mean ± SEM. * and # denotes $P<0.05$ when comparing AngII vs saline and WT vs Tg respectively (Two-way ANOVA with Holm-Sidak post hoc analysis).