m-Calpain Induction in Vascular Endothelial Cells on Human and Mouse Atheromas and Its Roles in VE-Cadherin Disorganization and Atherosclerosis

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Three major types of intercellular junctions have been identified in ECs: tight junctions, gap junctions, and adherence junctions (AJs). Tight junctions are present in up to 20% of the total number of junctional complexes in ECs, whereas AJs are found in ∼90% of them. AJs are composed of VE-cadherin, which forms Ca2+-sensitive and homophilic associations between adjacent ECs. Administration of anti-VE-cadherin neutralizing antibody to mice results in vascular hyperpermeability without modifying other types of interendothelial junctions; therefore, VE-cadherin-mediated AJs have an essential role in maintaining EC barrier functions. Bobryshev et al7 previously demonstrated that downregulation of VE-cadherin expression within intimal neovessels in human atherosclerotic lesions is accompanied by increased entry of immunocompetent cells into the intimal areas of neovascularization. Foteinos et al8 similarly described down-

Vascular endothelial cells (ECs) cover the luminal surface of blood vasculature in a wall-to-wall manner. This conformational aspect is known to act as a physical barrier against plasma constituents and immunocompetent cells, which is critically important to ensure vascular integrity. Dysfunction of the EC barrier during the early stage of atherosclerosis leads to leakage of low-density lipoprotein (LDL) and extravasation of monocytes into the vessel walls, thereby accelerating atherosclerosis.1,2

Background—Although dysfunction of VE-cadherin–mediated adherence junctions in vascular endothelial cells (ECs) is thought to be one of the initial steps of atherosclerosis, little is known regarding how VE-cadherin is disrupted during atherogenic development. This study focused on the role of calpain, an intracellular cysteine protease, in the proteolytic disorganization of VE-cadherin and subsequent progression of atherosclerosis.

Methods and Results—Increased expression of m-calpain was observed in aortic ECs in atherosclerotic lesions in humans and low-density lipoprotein receptor–deficient (ldlr–/-) mice. Furthermore, proteolytic disorganization of VE-cadherin was shown in aortic ECs in ldlr–/- and apolipoprotein E–deficient (apoE–/-) mice. Long-term administration of calpain inhibitors into these mice attenuated atherosclerotic lesion development and proinflammatory responses, as well as VE-cadherin disorganization, without normalization of plasma lipid profiles. Furthermore, in vivo transfection of m-calpain siRNA to ldlr–/- mice prevented disorganization of VE-cadherin and proatherogenic hyperpermeability in aortic ECs. Treatment of cultured ECs with oxidized LDL, lysophosphatidylcholine, or LDL pretreated with secreted phospholipase A2 led to the induction of m-calpain but not of μ-calpain, thereby eliciting selective m-calpain overactivation. These data suggest that lysophosphatidylcholine-induced m-calpain directly cleaves a juxtamembrane region of VE-cadherin, resulting in dissociation of β-catenin from the VE-cadherin complex, disorganization of adherence junctions, and hyperpermeability in ECs.

Conclusions—Subtype-selective induction of m-calpain in aortic ECs during atherosclerotic progression is associated with proteolytic disorganization of VE-cadherin and proatherogenic hyperpermeability in cells. Thus, a strategy to selectively inhibit m-calpain may be useful for the therapeutic treatment of patients with atherosclerosis. (Circulation. 2011;124:2522-2532.)

Key Words: atherosclerosis ■ endothelium ■ inflammation ■ cadherins ■ calpain
regulation of VE-cadherin and increased vascular permeabil-
ity in aortic intima in apolipoprotein E–deficient (apoE−/−) mice. It is therefore considered that VE-cadherin dysfunction
has a proatherogenic effect on vessels; however, the molec-
ular mechanisms of how AJs are disorganized during develop-
ment of atherosclerosis remain largely obscure.

It has recently been documented that the Ca2+–sensitive
intracellular cysteine protease calpain is capable of inducing
proteolysis and functional disruption of both the neuronal
(N)- and epithelial (E)-cadherin subtypes.9,10 Two ubiquitous
forms of calpain, μ- and m-calpain, require micromolar and
millimolar levels of Ca2+ for half-maximal activation, re-
spectively.11 Ubiquitous calpain is composed of a het-
erodimer of the common regulatory subunit, Capn1
and subtype-specific large subunits, Capn2, which serve as
μ- and m-calpain isoymes, respectively. Deficiency of the
Capn1 gene in mice results in a reduction in platelet
aggregation and clot retraction11; in contrast, Capn2-deficient
mice appear to be embryonically lethal.12 Similarly, defi-
cy of the Capn4 gene leads to an embryonically lethal
phenotype, which is accompanied by impaired cardiovascular
development and abnormal EC function.13 Our previous
study proposed that m-calpain, but not μ-calpain, dominantly
participates in shear stress–induced motility and barrier reg-
ulation in vascular ECs,14,15 indicating that m-calpain has a
fundamental role in the cardiovascular system and/or EC
functions. However, the roles of calpain in atherosclerosis
and VE-cadherin processing have not yet been fully estab-
lished. In this study, we addressed the question of whether
calpain proteolytically and functionally modifies the integrity
of endothelial AJs during the process of atherosclerosis. The
results showed that m-calpain is induced in ECs on human
and murine atherosclerotic lesions in aorta, which promotes
proteolytic disorganization of VE-cadherin and subsequent
proatherogenic hyperpermeability as a trigger of atheroscle-
rosis development.

Methods

An expanded discussion of material and methods is available in
the online-only Data Supplement.

Human Aorta

Atherosclerotic lesions in human aortic samples, which were col-
lected from 8 autopsy cases (5 male and 3 female patients; age,
26–72 years; the Table), were graded essentially on the basis of
American Heart Association criteria.16 Protein expression of
m-calpain in aortic segments was detected by conventional immuno-
histochemistry with anti–m-calpain antibody (Abcam, Cambridge,
MA). The study was approved by the Ethics Committee of Showa
University School of Medicine.

Mice

All procedures involving animals were performed according to
protocols approved by the faculties. LDL receptor–deficient
(ldlr−/−) and apolipoprotein E–deficient (apoE−/−) mice were
obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6
mice served as wild-type controls. Mice were fed chow (CRF-1;
Oriental Yeast, Tokyo, Japan) or a high-cholesterol diet (HCD;
CRF-1 supplemented with 1.4% cholesterol and 0.4% cholic acid;
Oriental Yeast) for 1 to 24 weeks starting at 8 weeks of age. Plasma
lipid profiles were determined by Oriental Yeast using standard
enzymatic methods. Plasma lysophosphatidylcholine (LPC) levels
were measured with an LPC assay kit (AZWELL, Osaka, Japan).

Administration of Drugs and Small
Interfering RNAs

For administering calpain inhibitors, control vehicle (3% dimethyl
sulfoxide), N-Acetyl-L-leucyl-L-leucyl-L-methioninal (ALLM; 5
mg/kg), or calpeptin (5 mg/kg) was dissolved in isotonic saline and
administered intraperitoneally to HCD-fed mice for 12 weeks at an
interval of 2 days. For in vivo small interfering RNAs (siRNA)
transfection, ldlr−/− mice were briefly anesthetized with diethyl
ether, followed by intravenous administration of siRNA against
m-calpain (10 μg per body) or nonsilencing control RNA (10 μg per
body) involving Transit-QR Hydrodynamic delivery solution (Mi-
rus Bio, Madison, WI), as previously described.15 The mice were fed
HCD from day 2 (2 days after siRNA administration) and were
euthanized on day 9. The nucleotide sequences of siRNA are
displayed in Table I in the online-only Data Supplement.

Immunohistochemistry and Atherosclerotic
Lesion Quantification

For immunohistochemical analysis in mice, the isolated aortic tree
and root were fixed in 4% paraformaldehyde in PBS; they were
subsequently frozen, sectioned at 6-μm thickness, and mounted on
glass slides as required. Target molecules were detected by the use of
immunofluorescence histochemistry or conventional immunohisto-
chemistry. For quantifying atherosclerotic lesions, paraformalde-
hyde-fixed aortic trees and roots were stained with Oil Red O in 60%
isopropanol for 30 minutes. Specimens were washed with 60% isopropanol
and photographed for calculation of positive areas with ImageJ software.

Table. Clinicopathological Information and m-Calpain Expression in Endothelial Cells in Human Aorta

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Atherosclerotic Grade</th>
<th>m-Calpain Expression in Endothelial Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>58</td>
<td>M</td>
<td>Gastric cancer</td>
<td>Mild to moderate</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>68</td>
<td>M</td>
<td>Skin cancer</td>
<td>Mild to moderate</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>62</td>
<td>M</td>
<td>Myocardial infarction</td>
<td>Moderate to severe</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>F</td>
<td>Scleroderma</td>
<td>Mild to moderate</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>26</td>
<td>M</td>
<td>Cerebral hemorrhage</td>
<td>Very mild</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>31</td>
<td>F</td>
<td>Anorexia nervosa</td>
<td>Mild</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>34</td>
<td>F</td>
<td>Anorexia nervosa</td>
<td>Mild</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>41</td>
<td>M</td>
<td>Acquired immunodeficiency syndrome</td>
<td>Mild</td>
<td>+</td>
</tr>
</tbody>
</table>

Atherosclerotic lesions were graded essentially on the basis of American Heart Association criteria. Very mild indicates that intimal
thickening is rarely observed. Mild, moderate, and severe are characterized by foam cell–enriched fatty streaks, advanced atheroma
that contains lipid core, and plaques with large calcification, respectively.
Aortic Permeability
For measuring aortic permeability, FITC-labeled BSA was intravenously administered to the mice before isolation of the vessels as described previously. One hour after administration of FITC-BSA, the circulation system was perfused with 4% paraformaldehyde in PBS. The aortic tree was then excised, and en face distribution of leaked FITC-BSA was imaged by confocal microscopy (LSM510; Carl Zeiss, Thornwood, NY). Aortic specimens were weighed and homogenized in 200 μL PBS, and the insoluble fraction was removed by centrifugation. Fluorescence intensity of the supernatant was monitored with a microplate reader (excitation, 485 nm; emission, 535 nm).

Real-Time Reverse-Transcriptase Polymerase Chain Reaction
Total RNA derived from the whole aorta or scaped aortic intimal cells was converted to cDNA that served as a template of polymerase chain reactions as previously described. Primer sequences used are shown in Table II in the online-only Data Supplement.

Cell Culture and Lysophospholipid Stimulus
Human umbilical vein ECs (HUVECs), which were purchased from Cambrex (Walkersville, MD), were cultured as previously described. Confluent cells from 4 to 7 passages were used in the experiments. For lysophospholipid stimulus, the cells were incubated with endothelial growth medium-2 (Cambrex) containing LPC (1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine), lysophosphatic acid (1-oleoyl-2-hydroxy-sn-glycero-3-phosphate), or sphingosine-1-phosphate at 37°C.

Immunoblotting, Immunoprecipitation, and Casein Zymography
Immunoblotting was performed as previously described. Primary antibodies used are listed in Table III in the online-only Data Supplement. Immunoprecipitation was conducted with Dynabeads protein G (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. For measuring calpain activity with casein zymography, HUVEC lysate was separated by electrophoresis with the use of 12% polyacrylamide gels containing 0.2% casein sodium. Subsequently, the gels were incubated with Ca2+-containing reaction buffer overnight at room temperature, followed by staining with Coomassie blue.

Preparation of Modified LDL
Human LDL (d=1.019–1.063 g/mL) was prepared as described previously. To prepare oxidized LDL, LDL (0.1 mg/mL) was incubated for 20 hours at 37°C with 5 μmol/L CuSO4, followed by the addition of 1 mmol/L EDTA and cooling. To prepare enzymatically hydrolyzed LDL, isolated LDL (500 μg/mL) was incubated with recombinant human secreted phospholipase A2, isoforms (group V, sPLA2-V; group X, sPLA2-X) at 1 μg/mL for 16 hours at 37°C as described previously. HUVECs were then incubated with 5-fold diluted sPLA2 reagents.

In Vitro siRNA
HUVECs were transfected with μ- or m-calpain siRNA (40 nmol/L) or control RNA (40 nmol/L) involving the siPORT NeoFX Transfection Agent (Ambion, Austin, TX) as previously described. Cells were subject to experiments 48 hours after transfection. The nucleotide sequences of the siRNA are shown in Table I in the online-only Data Supplement.

Measurement of EC Barrier Functions In Vitro
The permeability assay was performed as previously described by Patterson et al with a slight modification. HUVECs were cultured on cell culture inserts (0.4-μm pore; Millipore, Bedford, MA) coated with collagen type I. After 60 minutes of stimulation, Evans blue dye bound to BSA, which was prepared by supplementing Dulbecco modified Eagle medium containing 10% BSA with Evans blue dye at 1 mg/mL, was added to the upper chamber of the insert. Optical absorbance of the medium in the lower chamber was monitored at 595 nm using a microplate reader. The albumin flux (F) across the monolayer was determined from the following equation: F = ΔA/Δt, where A is optical absorbance in the lower chamber and t is time. For measuring transendothelial electric resistance, HUVECs were cultured on cell culture inserts (0.4-μm pore; Millipore) coated with collagen type I. Transendothelial electric resistance measurement was conducted with the EVOM2 Epithelial Voltohmeter (World Precision Instruments, Sarasota, FL).

DNA Constructs and Mutagenesis
The human VE-cadherin construct was digested at appropriate restriction sites and ligated in-frame into the N-terminus site of the pAcGFP1-N2 vector (Clontech, Palo Alto, CA). VE-cadherin deletion mutants were generated by using mutagenic primers and the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Primer sequences used in the recombination study are given in Table IV in the online-only Data Supplement. HUVECs were transfected with the expression vectors by lipofection involving Lipofectamine LTX regent (Invitrogen) and Nucleofector II (Lonza) and Amaza HUVEC Nucleofector kit (Lonza). Cells were subjected to experiments 24 hours after transfection. For evaluating VE-cadherin resistance to calpain-induced proteolysis, immunoprecipitated VE-cadherin was incubated with recombinant human μ-calpain or rat m-calpain (Calbiochem, San Diego, CA) at room temperature.

Data Analysis
Results are expressed as the mean±SEM. The Mann-Whitney U test was used to determine the statistical differences between the 2 groups. Multiple comparisons were conducted with non–repeated-measures 1-way or 2-way ANOVA followed by a posthoc Bonferroni or Dunnett test as appropriate. A value of P<0.05 was considered statistically significant.

Results
Subtype-Selective m-Calpain Induction in Aortic ECs Accelerates Atherosclerosis Progression
We first investigated m-calpain expression in the aortic segments derived from 8 patients with various grades of atherosclerotic lesions (the Table). Immunohistochemistry showed that m-calpain expression was prominent in particular in ECs on advanced atheroma in aorta (Figure 1A), whereas relatively weak m-calpain expression was noted in other component cells in the lesion. In the atheroma-free aorta, m-calpain immunoreactivity was noted in intimal ECs and other vascular wall cells in aorta was much weaker (Figure 1B) than that in ECs on advanced atheroma (Figure 1A).

We then confirmed systemic expression of the 2 ubiquitous calpain subtypes, μ- and m-calpains, in the chow- or HCD-fed ldlr+/- mice by real-time reverse-transcriptase polymerase chain reaction (Figure 2A). Although the expression levels of μ-calpain were reduced in the spleen during 12 weeks of HCD feeding, those of m-calpain were significantly increased in the aorta and lung compared with those in chow feeding (P<0.05). Confocal immunohistochemistry showed that the density of m-calpain–positive ECs was clearly increased in aortic intima in HCD-fed ldlr+/- mice compared with chow-fed mice (Figure 2B). Intense staining was restricted to ECs in superficial intima in the initial fatty-streak lesions and in the advanced lesions (Figure 2B), but it was not restricted to the elastin-enriched medial regions or plaque.
interior. Similar EC-specific localization of m-calpain was observed in lung in HCD-fed ldlr/H11002/H11002 mice. Real-time reverse-transcriptase polymerase chain reaction analysis showed that there was a marked increase in the expression of intimal m-calpain, but not /H9262-calpain, during 6 weeks of HCD feeding (Figure 2C).

Oxidized LDL and sPLA2-modified LDL are known to trigger proatherogenic responses in various cell types, including ECs; therefore, the effects of these modified LDLs on the expression of calpains were tested in cultured ECs (Figure 2D). Accordingly, the expression of m-calpain, but not /H9262-calpain, was dramatically elevated by administration of sPLA2-V– or sPLA2-X–modified LDL compared with the enzyme-alone groups, which increased m-calpain expression only modestly, and with native LDL, which had no influence on the expression. Similarly, subtype-specific m-calpain induction was also observed in HUVECs stimulated with oxidized LDL. Our preliminary data showed that the antioxidants /N-acetylcysteine or superoxide dismutase did not attenuate the oxidized LDL–induced m-calpain induction, probably indicating that the induction was not due mainly to oxidative stress (data not shown).

Because proatherogenic lysophospholipids are reportedly accumulated in modified LDLs, we investigated whether lysophospholipids directly induce calpain expression in cultured ECs (Figure 2E). Exposure of HUVECs to LPC resulted in an increase in expression levels of m-calpain protein above 1 µmol/L without altering those of /H9262-calpain (Figure 2E), which was accompanied by selective m-calpain overactivation (Figure 2F). In contrast, lysosphosphatic acid and sphingosine-1-phosphate failed to induce m-calpain in ECs (Figure 2E). In agreement with the effect of LPC to induce m-calpain, sPLA2-treated LDL and oxidized LDL accumulated ~300 to 500 nmol LPC per 1 mg protein (Figure I in the online-only Data Supplement).

The therapeutic effects of long-term administration of calpain inhibitors on atherosclerosis were then evaluated. Administration of each calpain inhibitor, calpeptin or ALLM, to ldlr−/− mice led to suppression of HCD-induced atherosclerosis in the aortic tree and root (Figure 3A, left), whereas plasma levels of LPC and total, LDL, and high-density lipoprotein cholesterol were unaffected (Table V in the online-only Data Supplement). In addition, calpeptin displayed a similar therapeutic effect on atherosclerosis in HCD-fed apoE−/− mice (Figure 3A, right). Moreover, mRNA expression levels of the proinflammatory markers, including nuclear factor-κB, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, E-selectin, tumor necrosis factor-α, interleukin-1β, and interleukin-6, as well as the macrophage marker CD68, in the aortas in ldlr−/− mice were significantly decreased by the inhibitors (Figure 3B). Furthermore, infiltration of macrophages into the vascular wall in the hypercholesterolemic mice, as assessed by immunohistochemistry with MOMA-2 antibody, was significantly decreased by the inhibitor treatment (Figure 3C).

Figure 1. Expression of m-calpain in endothelial cells (ECs) on atherosclerotic lesions in human aorta. Elastica van Gieson (EVG; left), hematoxylin and eosin (HE; center), and immunohistochemical images of m-calpain (m-cal; right) are depicted. A, Prominent expression of m-calpain in ECs on an advanced atherosclerotic plaque derived from a male patient who died of myocardial infarction at 62 years of age (patient 3). B, m-Calpain expression in atheroma-free aorta derived from a male patient who died of cerebral hemorrhage at 26 years of age (patient 5).

m-Calpain Mediates VE-Cadherin Cleavage and Proatherogenic Hyperpermeability

Immunoblotting with anti–VE-cadherin antibody (H-72) indicated that 2 major bands, 135 kDa (intact VE-cadherin) and its ~90-kDa fragment, were found in the whole aortic lysate. Furthermore, the expression ratio of the ~90- to 135-kDa band was significantly elevated in HCD-fed ldlr−/− mice within 6 weeks of diet initiation compared with that in the chow-fed mice (P<0.05; Figure 4A). VE-cadherin cleavage, which was observed in aorta in HCD-fed ldlr−/− or apoE−/− mice, was prevented by the administration of calpeptin or ALLM (Figure 4B). It is noteworthy that the calpain inhibi-
tion resulted in a parallel reduction in proteolysis of α-spectrin, a well-known calpain substrate. Immunohistochemistry showed that the VE-cadherin disorganization, which was observed in aortic ECs in the hypercholesterolemic mice after treatment with dimethyl sulfoxide controls, was restored by inhibition of calpain (Figure 4C).

The effects of in vivo siRNA transfection on VE-cadherin cleavage and initial atherosclerotic hyperpermeability were investigated. Hydrodynamic transfection of m-calpain siRNA selectively diminished the expression of aortic m-calpain protein by 65.1±0.1% (mean±SEM; n=4) at day 2 (2 days after transfection) compared with mice that received control RNA (Figure II in the online-only Data Supplement). Although this m-calpain silencing was weakened at day 9, m-calpain expression remained at a 40.1±0.1% lower level (mean±SEM; n=8) at this time point (Figure 5A), indicating that the effect of siRNA was sustained at least for 9 days. Cleavage of aortic VE-cadherin and α-spectrin was markedly inhibited by siRNA silencing of m-calpain at day 9 (Figure 5A) without normalization of plasma lipid profiles (Table V in the online-only Data Supplement). An in vivo permeability assay showed that ldlr-/- mice fed HCD for 7 days displayed leakage of FITC-BSA, particularly in the inferior aspect of the aortic arch, which was not observed in the chow-fed mice (Figure 5B). Although administration of control RNA did not influence proatherogenic hyperpermeability in the aorta, it was largely attenuated by m-calpain silencing.

Figure 2. Selective induction of m-calpain in endothelial cells (ECs) in hypercholesterolemic mice. A, Tissue distribution of mRNAs for μ-calpain (Cal) and m-calpain in chow- or high-cholesterol diet (HCD)-fed ldlr-/- mice. n=8. *P<0.05 by 2-way ANOVA and Bonferroni test. B, EC-specific induction of m-calpain in aorta, liver, and lung in HCD-fed ldlr-/- mice. Immunohistochemical distribution of VE-cadherin in liver and lung was represented as a marker of ECs. n=3. C, Temporal changes in μ-calpain or m-calpain mRNA expression in aortic intima. n=6 to 8. *P<0.05 vs chow by 2-way ANOVA and the Bonferroni test. D, Modified low-density lipoprotein (LDL) induces m-calpain protein expression in human umbilical vein ECs (HUVECs). The cells were subjected to secreted phospholipase A₂ (sPLA₂) isozymes alone (V and X), sPLA₂-modified LDL (V+LDL and X+LDL), oxidized (Ox) LDL, or native LDL (LDL) for 1 hour. n=4. *P<0.05 vs untreated by 1-way ANOVA followed by the Dunnett test. E, Lysophosphatidylcholine (LPC) induces m-calpain protein expression in HUVECs. The cells were treated with the indicated concentrations of lysophospholipids for 1 hour. n=4. *P<0.05 vs untreated by 1-way ANOVA followed by the Dunnett test. F, Subtype-selective activation of m-calpain in LPC-stimulated HUVECs. The cells were incubated with 10 μmol/L LPC for 1 hour. n=4. *P<0.05 by Mann-Whitney U test.
LPC-Induced m-Calpain Cleaves VE-Cadherin, Leading to EC Barrier Dysfunction

Exposure of HUVECs to LPC, but not to lysophosphatidic acid or sphingosine-1-phosphate, resulted in an increasing cleavage of VE-cadherin above 1 μmol/L (Figure 6A). This cleavage, which was detected similarly in ionomycin-stimulated HUVECs, was temporally correlated with m-calpain induction (Figure III in the online-only Data Supplement). The calpain inhibitor calpeptin, the intracellular Ca\(^{2+}\) chelator BAPTA/AM, the phospholipase C inhibitor U73122, and the extracellular signal-related kinase (ERK) kinase inhibitor U0126 were all effective for cancellation of LPC-induced VE-cadherin cleavage (Figure 6B). Immunoprecipitation experiments showed that treatment of HUVECs with LPC resulted in dissociation of β-catenin from VE-cadherin, an effect that was clearly cancelled by administration of calpeptin (Figure 6C).

Figure 3. Amelioration of atherosclerosis by long-term administration of calpain inhibitors. The high-cholesterol diet (HCD)-fed ApoE\(^{-/-}\) and apoE\(^{-/-}\) mice received vehicle (dimethyl sulfoxide [DMSO]) and calpain inhibitors (calpeptin [Calp] and/or ALLM) at 5 mg/kg for 3 days throughout 12 weeks of the diet. A, Calpain inhibitor trials suppress Oil red O-positive atherosclerotic lesions in the aortic tree and root. B, Calpain inhibitor trials prevent mRNA induction of proinflammatory markers in the aorta. C, Calpain inhibitor trials reduce macrophage contents in atherosclerotic lesions in the aortic root. NF-κB indicates nuclear factor-κB; TNF-α, tumor necrosis factor-α; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; and VCAM-1, vascular cell adhesion molecule-1.
We further conducted siRNA-based inhibition experiments in HUVECs (Figure 6D). LPC-induced induction of m-calpain was observed in the control and /H9262-calpain knockdown cells but not in the m-calpain knockdown cells. LPC-induced VE-cadherin cleavage was detected equally in the control and /H9262-calpain knockdown cells. However, m-calpain knockdown cells did not respond to the stimuli. Moreover, although LPC administration facilitated albumin permeability (a marker of EC barrier function) identically in control and /H9262-calpain knockdown cells, it failed to do so in m-calpain knockdown cells (Figure 6E). Furthermore, transfection with the full-length human m-calpain gene in HUVECs resulted in a promotion of VE-cadherin cleavage and disorganization of AJs (Figure IV in the online-only Data Supplement).

**m-Calpain Directly Cuts VE-Cadherin at Juxtamembrane Regions**

In vitro enzymatic experiments showed that recombinant m-calpain, but not /H9262-calpain, potently cleaved immunoprecipitated VE-cadherin (Figure 7A). Antibody-based epitope mapping indicated that the calpain cleavage site might reside in amino acid residues 651 to 722, a juxtamembrane region (Figure V in the online-only Data Supplement). To gain support of the epitope mapping, the expression vectors encoding deleted VE-cadherin constructs lacking 5 amino acid residues—671 through 675 (671 /H9004 5), 681 through 685 (681 /H9004 5), or 691 through 695 (691 /H9004 5)—were constructed and then transiently transfected into HUVECs. The expression levels of tagged VE-cadherin in cells transfected with wild-type or either of the deletion mutants were similar to those of endogenous VE-cadherin (Figure VI in the online-only Data Supplement). Enzymatic study showed that 2 VE-cadherin mutant proteins, 671 /H9004 5 and 681 /H9004 5, were proteolyzed by recombinant m-calpain in an identical fashion to wild-type VE-cadherin, whereas the 691 /H9004 5 mutant exhibited resistance to m-calpain–induced proteolysis (Figure 7B).

Confocal microscopic analysis showed that transfection of HUVECs with wild-type or mutant VE-cadherin led to peripheral localization of the green fluorescent protein fusion protein under unstimulated conditions (Figure 7C). Treatment of cells transfected with wild-type or the 681 /H9004 5 mutant with LPC led to breakdown of AJs, whereas AJs in the 691 /H9004 5 mutant were not disorganized by LPC stimulus. Furthermore, LPC-induced decreases in transendothelial electric resistance,
which reflects AJ disorganization, were observed both in wild-type and 681Δ5 mutant but not in 691Δ5 mutant. Finally, although LPC administration facilitated albumin permeability equivalently in wild-type or the 691/H9004 5 mutant but not in 681/H9004 5 mutant, the 691Δ5 mutant did not respond to the stimuli (Figure 7D).

Discussion

Our findings showed that the subtype-selective induction of m-calpain detected in aortic ECs in hypercholesterolemic mice participates in proteolytic disorganization of VE-cadherin (Figure 7E). This observation is reminiscent of atherosclerosis in humans because EC-specific induction of m-calpain also occurs in atherosclerotic lesions in human aorta (Figure 1 and the Table). Considering that downregulation of VE-cadherin reportedly promotes extravasation of immunocompetent cells or macromolecules into the vascular wall, m-calpain–induced disorganization of VE-cadherin in aortic ECs may accelerate the progression of atherosclerotic lesions.

The proatherogenic role of μ-calpain has been predicted. ATP-binding cassette transporter A1 (ABCA1) in macrophages and hepatocytes is reportedly inactivated by μ-calpain–induced proteolysis. Hepatic ABCA1 degradation observed in cholesterol-fed rabbits is decreased by the antioxidative hypolipidemic agent probucol, which leads to increased high-density lipoprotein biogenesis and thereby ameliorates atherosclerosis. This finding suggests the potential contribution of μ-calpain to ABCA1 degradation during atherogenesis. However, we believe that this is not the case in hypercholesterolemic mice because direct inhibition of calpain by pharmacological inhibitors or siRNA attenuated the progression of atherosclerotic lesion in the mice without normalization of plasma cholesterol and high-density lipoprotein levels (Table V in the online-only Data Supplement). Although the role of m-calpain subtype in the atherogenic process has not been previously investigated, our present data indicated that m-calpain is preferentially induced in ECs on atheroma rather than other component cells in atherosclerotic lesions (eg, smooth muscle cells or foam cells; Figures 1 and 2). Thus, it is likely that m-calpain in vascular ECs has a key role in atherogenesis.

The present in vitro data further suggest, for the first time, that VE-cadherin is directly proteolyzed by m-calpain at a juxtamembrane region (Figure 7). N-cadherin appears to be cleaved by calpain at 4 or more additional regions of the cytoplasmic domain, including juxtamembrane regions. Similarly, E-cadherin is reportedly proteolyzed by calpain at its juxtamembrane region. Therefore, the calpain–induced cleavage sites in VE-cadherin resemble those in other cadherin families. The cytoplasmic domain of VE-cadherin contains a C-terminal site for binding to either β-catenin or γ-catenin (plakoglobin) and a juxtamembrane site for binding to p120-catenin. In addition, β-catenin and γ-catenin are associated with α-catenin, which provides physical linkage of the cadherin complex to actin cytoskeleton. Navarro et al demonstrated that a mutant VE-cadherin lacking the C-terminal tail (AA702–784) results in dissociation of α- and β-catenin from VE-cadherin and that Chinese hamster ovary cells expressing truncated VE-cadherin display robust macromolecule permeability compared with those expressing nontruncated VE-cadherin. This is consistent with the present results showing that m-calpain–induced VE-cadherin cleavage in the juxtamembrane domain (adjacent to AA690–695

Figure 5. m-Calpain (Cal) accelerates initial proatherogenic hyperpermeability. A, m-Calpain-knockdown attenuates VE-cadherin (VE-cad) and α-spectrin cleavages in aorta in high-cholesterol diet (HCD)–fed ldlr−/− mice. The top diagram shows the experimental protocol. Immunoblots of each protein in the mice that received control RNA (CTL) or m-calpain siRNA (Si(m); left) are quantified by a densitometer (middle). The right graph indicates the ratio of 90- to 135-kDa VE-cadherin and the ratio of 150- to 220-kDa α-spectrin. n = 7. *P < 0.05 vs control by Mann-Whitney U test. B, In vivo transfection of m-calpain siRNA attenuates proatherogenic hyperpermeability. Photographs show the en face distribution of leaked FITC-BSA in representative aortic arch segments. Arrows represent FITC-BSA leakage. The right graph indicates quantification of leaked FITC-BSA. n = 5 to 7. *P < 0.05; #P < 0.05 by 1-way ANOVA followed by the Bonferroni test.
residues) leads to β-catenin/VE-cadherin dissociation and barrier dysfunction in ECs (Figures 6 and 7).

We now show that LPC, which can reach a nearly equal amount of intact phosphatidylcholine (a major phospholipid component) in oxidized LDL, triggers the expression and proteolytic conversion of m-calpain in ECs (Figures 2 and 6). LPC reportedly decreases nitric oxide production and increases adhesion molecule expression in ECs and facilitates EC barrier dysfunction through protein kinase C/RhoA-mediated cytoskeletal signaling, indicating its proatherogenic role in ECs. Furthermore, treatment of cultured ECs with LPC appears to induce Ca²⁺-dependent calpain activation and ERK activation. It is noteworthy that ERK activation is capable of sensitizing m-calpain to Ca²⁺. Because such biochemical sensitization is functional in living cells, m-calpain can be activated intracellularly.

Although LPC is abundantly present in plasma, local production of LPC from LDL by sPLA₂ enzymes in the arterial wall has been considered to play an important role in the process of atherosclerosis, and an sPLA₂ inhibitor is now under clinical trial for the treatment of atherosclerosis. Indeed, LDL treated with sPLA₂ enzymes contained large amounts of LPC (Figure I in the online-only Data Supplement), and sPLA₂-treated LDL resulted in induction of m-calpain in ECs (Figure 2D). Considering that sPLA₂ is increased in the atherosclerotic lesions, arterial hydrolysis of LDL by sPLA₂ (and

Figure 6. m-Calpain (Cal) elicits VE-cadherin (VE-cad) cleavage, β-catenin/VE-cadherin dissociation, and hyperpermeability in cultured endothelial cells (ECs). The cells were subjected to lysophosphatidylcholine (LPC) or other lysophospholipids for 1 hour. A, LPC accelerates VE-cadherin cleavage in human umbilical vein ECs. The graph represents the ratio of 90- to 135-kDa VE-cadherin. n=4, *P<0.05 vs untreated by 1-way ANOVA followed by the Dunnett test. B, Pharmacological evaluation of the mechanisms underlying VE-cadherin cleavage. Calpeptin (10 μmol/L; calp), BAPTA/AM (50 μmol/L; BA), U73122 (10 μmol/L; U73), or U0126 (5 μmol/L; U01) was supplemented to the culture medium from 30 minutes before application of 10 μmol/L LPC. n=4, *P<0.05 vs untreated, #P<0.05 vs control by 1-way ANOVA followed by the Bonferroni test. C, Calpain inhibition attenuates β-catenin/VE-cadherin dissociation induced by 10 μmol/L LPC. Calpeptin (10 μmol/L) was administered to the cells in a manner similar to that in B. n=4, *P<0.05 vs untreated by 1-way ANOVA followed by the Dunnett test. D, Knockdown of m-calpain weakens VE-cadherin cleavage induced by 10 μmol/L LPC. The cells were transiently transfected with control RNA (CTL) or m- or m-calpain siRNA. n=5, *P<0.05 vs control by 2-way ANOVA and the Dunnett test. E, Knockdown of m-calpain inhibits EC hyperpermeability induced by 10 μmol/L LPC. n=6. *P<0.05 by 2-way ANOVA and the Bonferroni test. IP indicates immunoprecipitation; S1P, sphingosine-1-phosphate.
thereby local elevation of LPC) in developing plaques may facilitate the expression of m-calpain and subsequent disorganization of AJs in ECs in vivo.

**Conclusions**

The present study offers novel insights into the proatherogenic role of m-calpain in ECs. Furthermore, these findings provide a molecular basis of VE-cadherin disorganization during atherosclerosis development. m-Calpain is induced in the proatherogenic arterial wall in an EC-specific manner; thus, it can be regarded as a unique molecular target for controlling atherosclerosis. Selective m-calpain inhibitor may be highly desirable to achieve a better therapeutic outcome.

**Sources of Funding**

This study was supported in part by a Showa University special grant-in-aid for an innovative collaborative research project (to H. Ohata), KAKENHI from the Japan Society for the Promotion of Science (to Dr T. Miyazaki and H. Ohata), and a grant-in-aid from the Japan Health Sciences Foundation (to H. Ohata).

**Disclosures**

None.

**References**

One of the earliest pathological features of atherosclerosis is barrier dysfunction in vascular endothelial cells, which triggers the infiltration of monocytes/macrophages or plasma active constituents into subendothelial space, allowing further development of atherosclerosis: a report from the Committee on Vascular Lesions of the Circulation December 6, 2011

CLINICAL PERSPECTIVE

One of the earliest pathological features of atherosclerosis is barrier dysfunction in vascular endothelial cells, which triggers the infiltration of monocytes/macrophages or plasma active constituents into subendothelial space, allowing further increase in the atherosclerosis susceptibility in the large artery. Although VE-cadherin-mediated adherence junctions, a dominant determinant of endothelial cell barrier functions, are known to decay in the early phase of atherosclerosis, the molecular mechanism underlying this disorder remains unknown. Here, we show that m-cadlania, an intracellular cysteine protease, is induced in vascular endothelial cells in murine and human atherosclerotic aortas. Furthermore, this study provides direct evidence that m-cadlania proteolytically cleaves VE-cadherin at its juxtamembrane regions, leading to endothelial cell barrier dysfunction. Importantly, atherosclerosis in mouse models is ameliorable by calpain inhibition trials. Thus, m-cadlania can be regarded as a unique molecular target for controlling atherosclerosis. Subtype-selective m-cadlania inhibitor may be highly desirable to achieve a better therapeutic outcome.
m-Calpain Induction in Vascular Endothelial Cells on Human and Mouse Atheromas and Its Roles in VE-Cadherin Disorganization and Atherosclerosis

Takuro Miyazaki, Yoshitaka Taketomi, Masafumi Takimoto, Xiao-Feng Lei, Shigeko Arita, Joo-ri Kim-Kaneyama, Satoru Arata, Hisayuki Ohata, Hidekazu Ota, Makoto Murakami and Akira Miyazaki

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SUPPLEMENTAL MATERIAL

m-Calpain induction in vascular endothelial cells on human and mouse atheromas and its roles in VE-cadherin disorganization and atherosclerosis

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Short title: Miyazaki et al m-Calpain proteolysis of VE-cadherin


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Supplemental methods

Reagents

Lysophosphatidylcholine (1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine) and lysophosphaticid acid (1-oleoyl-2-hydroxy-sn-glycero-3-phosphate) were purchased from Avanti Polar Lipids (Birmingham, AL). Sphingosine 1-phosphate was obtained from Cayman Chemical (Ann Arbor, MI). U73122, Ly294002 and ALLM were purchased from Sigma (St. Louis, MO). BAPTA/AM was purchased from Invitrogen (Carlsbad, CA). All other chemicals were commercial products of the highest available grade of purity.

Immunohistochemistry

To conduct en-face immunohistochemical analysis, isolated aortic specimens were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) followed by permeabilization with 0.4% triton X-100 or 0.4% sodium dodecyl sulfate (SDS) in PBS. The specimens were then blocked with 10% bovine serum albumin (BSA), subsequently incubated with primary antibody raised against VE-cadherin (Alexis, San
Diego, CA) or m-calpain (Sigma). The specimens were labeled with fluorescent-labeled secondly antibody and 4',6-diamidino-2-phenylindole (DAPI). For acquiring en-face immunohistochemical images, aortic specimens were appressed intimal side down to a coverslip mounted on the stage of inverted confocal microscopy (LSM510; Carl Zeiss, Thornwood, NY) equipped with a 40 x oil immersion objective lens (1.3 NA) and appropriate filter sets. Series of Z-slice images of the aortic intima were collected, and were appropriately reconstructed in 3D image using Imaris (Bitplane AG, Zurich, Switzerland) and ImageJ software (National Institutes of Health, Rockville, MD). To conduct sectional analysis, PFA-fixed aortic root specimens were frozen, sectioned at 6-µm thickness and mounted on glass slides. The specimens were then blocked by 2.5% BSA, subsequently incubated with primary antibody raised against mouse macrophages/monocytes (MOMA-2; AbD Serotec, Oxford, UK) or m-calpain (Sigma). For immunofluorescence histochemistry, the specimens were labeled with fluorescent-labeled secondly antibody and DAPI, followed by imaging by confocal microscopy (LSM510; Carl Zeiss). For conventional immunohistochemistry, the specimens were labeled with peroxidase-labeled secondly antibody, and were detected
by using 3,3'-diamino-benzidine tetrahydrochloride (DUB) kit (DAKO, Carpintería, CA). Following counterstaining with Mayer’s hematoxylin solution (Sigma), immunohistochemical images were obtained by stereomicroscope (Carl Zeiss). The positive areas in the specimens were calculated using ImageJ software.

**Green fluorescent protein (GFP)-VE-cadherin dynamics**

For measuring GFP-VE-cadherin dynamics, wild-type cells or deletion mutants cultured on collagen-coated glass-bottom culture dish were mounted on a confocal microscopy (LSM510; Carl Zeiss) equipped with a 40 x oil immersion objective lens (1.3 NA) and appropriate filter sets. The cells were cultured in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum (FBS), and were kept at 37°C during fluorescence measurements. To avoid excess laser irradiation, time-series images were obtained in a 10-min interval.

**Immunoprecipitation**
Human umbilical vein endothelial cells (HUVECs) were lysed on ice with lysis buffer (50 mmol/L Tris-HCl; pH 7.5, 120 mmol/L NaCl, 0.5% Nonidet P-40, 40 µmol/L phenylmethylsulfonyl fluoride, 50 µg/mL leupeptin, 50 µg/mL aprotinin, 200 µmol/L sodiumorthovanadate, 1 mmol/L EGTA). For IgG capture, anti-β-catenin antibody (AnaSpec, San Jose, CA; 1.5 µg/reaction) or anti-GFP antibody (Clontech, Palo Alto, CA; 2 µg/reaction) were rotated with Dynabeads protein G (Invitrogen; 25 µL/reaction) in citrate phosphate buffer (24.5 mmol/L citric acid, 51.7 mmol/L dibasic sodium phosphate; pH 5.0) for 1hr at room temperature. Following washing three times with citrate phosphate buffer containing 0.1% Tween-20, the IgG-conjugated beads were magnetically separated and supplemented to the HUVEC lysate, and were subsequently rotated for 1hr at room temperature. The beads were then washed three times by citrate phosphate buffer containing 0.1% Tween-20, and were eluted by boiling in sodium dodecyl sulfate (SDS) sample buffer (25 mmol/L Tris-HCl:pH 6.5, 5% glycerol, 1% SDS, 0.05% bromophenol blue, 3% 2-mercaptoethanol). Eluted proteins were analyzed by immunoblotting.
Casein zymography

For measuring calpain activity, HUVECs were lysed on ice with lysis buffer (50 mmol/L Tris-HCl; pH 7.5, 120 mmol/L NaCl, 0.5% Nonidet P-40, 40 µmol/L phenylmethylsulfonyl fluoride, 50 µg/mL leupeptin, 50 µg/mL aprotinin, 200 µmol/L sodiumorthovanadate, 1 mmol/L EGTA). Supernatants of the lysates were then obtained by centrifugation and protein contents in the supernatants were quantified by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Subsequently, protein aliquots were separated by electrophoresis in 12% polyacrylamide gel containing 0.2% casein using an imidazole/HEPES electrophoresis buffer (43 mmol/L imidazol and 35 mmol/L HEPES). Subsequently, the gels were incubated with Ca\(^{2+}\)-containing reaction buffer (20 mmol/L Tris-HCl: pH7.4, 10 mmol/L dithiothreitol (DTT), 4 mmol/L CaCl\(_2\)) overnight at room temperature followed by stained with Coomassie blue.

In vitro digestion of VE-cadherin by recombinant m-calpain

For evaluating resistance of VE-cadherin to calpain-induced proteolysis, immunoprecipitated VE-cadherin derived from wild-type ECs or mutants was eluted by
supplementing citrate buffer (0.1 mol/L citric acid: pH 3.0) into the beads. Then the eluted VE-cadherin was incubated with recombinant μ- or m-calpain (Calbiochem, San Diego, CA) at 5.5 to 16.5 µg/mL in the reaction buffer (375 mmol/L HEPES: pH 7.4, 12.5 mmol/L DTT, 1.25 mmol/L CaCl₂, 12.5% glycerol) at room temperature for 20 to 40 min. Subsequently, enzymatic reactions were terminated by supplementing x5 SDS sample buffer (125 mmol/L Tris-HCl: pH 6.5, 25% glycerol, 5% SDS, 0.25% bromophenol blue, 15% 2-mercaptoethanol) into the reactants. VE-cadherin cleavage was analyzed by immunoblotting.

**Wild-type and mutant VE-cadherin construct**

Wild-type full-length human VE-cadherin construct was amplified by RT-PCR from HUVECs, and subcloned into pCR2.1-TOPO vector by using TOPO TA cloning kit (Invitrogen). C-terminus stop codon of the construct was then removed by restriction enzyme-based recombination. DNA sequence of the construct was verified employing ABI prism 310 genetic analyzer (Applied Biosystems, Foster City, CA) by using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The wild-type
construct was subsequently digested at appropriate restriction sites, and was ligated in-flame into N-terminus site of pAcGFP1-N2 vector (Clontech). VE-cadherin deletion mutants were generated by using mutagenic primers and QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to manufacture’s instructions. Site-directed deletion of the construct was confirmed by DNA sequencing. VE-cadherin expression vector (1 - 2.5 µg) was transiently transfected into HUVECs by lipofection involving Lipofectamine LTX regent (Invitrogen) or electroporation using Nucleofector II (Lonza, Walkersville, MD) and Amaxa HUVEC Nucleofector kit (Lonza). Cells were subjected to experiments 24 h following transfection. Primers using recombination study were indicated in Supplementary table S5.
Supplemental figure legends

Supplemental figure S1 LPC is accumulated in the sPLA₂-hydrolyzed and oxidized LDLs. To prepare oxidized LDL (ox-LDL), LDL (0.1 mg/ml) was incubated for 20 h at 37°C with 5 µmol/L CuSO₄, followed by the addition of 1 mmol/L EDTA and cooling. To prepare enzymatically-hydrolyzed LDL, isolated LDL (500 µg/mL) was incubated with recombinant sPLA₂ isozymes (group V and group X sPLA₂; V+LDL and X+LDL, respectively) at 1 µg/mL for 16 hr at 37°C. Following stimulation of HUVECs with the modified LDLs, LPC in culture medium was quantified by LPC assay kit (AZWELL, Osaka, Japan). Values are indicated as mean ± SEM (n=4 independent experiments; *p<0.05 vs LDL).

Supplemental figure S2 Hydrodynamic siRNA transfection suppressed aortic m-calpain expression in ldlr−/− mice. Total RNA or protein lysate were derived from whole aorta in control and siRNA mice 2 days following siRNA administration (CTL and Si(m), respectively). (A) Diagram shows experimental protocol. (B) mRNA expression levels of μ- and m-calpain in control and siRNA mice were determined by qPCR (n=4 animals; *p<0.05 vs control). (C) Protein expression levels of m-calpain in control and siRNA mice were measured by immunoblotting (n=4 animals; *p<0.05 vs
Supplemental figure S3 (A) Temporal changes in calpain mRNA expression levels in LPC-stimulated HUVECs. LPC were subjected to the cells at 10 µmol/L for indicated time periods. µ- and m-calpain expression levels (µ- and m-Cal, respectively), which were normalized by β-actin, are presented. n=3; *p<0.05 vs 0 min. (B) Temporal changes in calpain protein expression in LPC-stimulated HUVECs. The cells were subjected to 10 µmol/L LPC for the indicated time periods. n=4; *p<0.05 vs 0 min. (C) Temporal changes in VE-cadherin proteolysis in LPC-stimulated HUVECs. The cells were incubated with 10 µmol/L LPC for indicated time periods. Graph represents ratio of 90kDa to 135kDa VE-cadherin (VE-cad). n=3; *p<0.05 vs 0 min. (D) VE-cadherin was proteolyzed in ionomycin-stimulated HUVECs. The cells were subjected to 10 µmol/L ionomycin for the indicated time periods. n=3; *p<0.05 vs 0 min.

Supplemental figure S4 Human m-calpain construct, which was purchased from Open Biosystems (Huntsville, AL), was digested at appropriate restriction sites, and was ligated in-flame into pcDNA3.1 vector. m-Calpain expression vector (0.2 µg) were transiently transfected into HUVECs involving Lipofectamine LTX regent (Invitrogen).
Cells were subjected to subsequent experiments 48 h following transfection. (A) VE-cadherin cleavage in m-calpain-overexpressing ECs. The cells were transfected with empty vector (vector) or m-calpain expression vector (m-Cal OE). n=3; *p<0.05. (B) VE-cadherin disorganization in m-calpain-overexpressing ECs. The GFP vector (Clontech) was co-transfected as a marker of transfected cells. Insets indicate GFP expression in the cells. Images are representative of four independent experiments.

Supplemental figure S5 Antibody-based epitope mapping of VE-cadherin. In order to map the m-calpain-induced cleavage site of VE-cadherin, EC lysates were immunoblotted using various antibodies that recognize different epitopes. As a result, the N-terminal antibody ALX-210 recognized both intact 135kDa and ≈90kDa fragment of VE-cadherin. The cytoplasmic antibody H-72, which is mapped to residues 651–722, recognized intact, ≈90kDa and ≈35kDa fragment; in contrast, the C-terminal antibody F-8 recognized intact and ≈35kDa fragment. Thus, the calpain cleavage site is predicted to reside in amino acid residues 651–722, a juxtamembrane region.
Supplemental figure S6  Immunoblot detection of endogenous and exogenous VE-cadherin in HUVEC transfectants. Immunoblot with anti-VE cadherin shows that the expression levels of tagged VE-cadherin or its mutant proteins were similar to that of endogenous VE-cadherin (top panel). Expression of tagged VE-cadherin was evaluated by immunoblot with anti-tag (GFP) antibody (middle panel). Equal sample loading was confirmed by immunoblot for β-actin (bottom panel). Blots are representative of four independent experiments.
Supplemental figure S1

![Graph showing LPC contents (nmol/mg LDL) for LDL, V+LDL, X+LDL, and Ox-LDL. The graph includes error bars.]

- LDL: Low LPC content
- V+LDL: Moderate LPC content
- X+LDL: High LPC content
- Ox-LDL: High LPC content

Significance indicated by asterisks: * denotes a significant difference.
Supplemental figure S2

A

Control or siRNA transfection → Sacrifice

2 weeks

Chow

2 days

B

mRNA expression (fold change)

0 0.5 1 1.5

Cont Si(m)

μ-Cal m-Cal

C

m-Cal/actin (fold change)

0 0.5 1 1.5

Cont Si(m)

m-Cal Actin

*
Supplemental figure S3

A) LPC (min)

µ-Cal -

m-Cal -

Actin -

B) LPC (min)

µ-Cal -

m-Cal -

Actin -

C) LPC (min)

VE-cad - 90kDa -

Actin -

D) Ionomycin (min)

VE-cad - 90kDa -

Actin -
Supplemental figure S5

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<td>a</td>
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<tr>
<td>b</td>
<td>H-72</td>
<td>651-722</td>
</tr>
<tr>
<td>c</td>
<td>F-8</td>
<td>735-784</td>
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Supplemental figure S6

Exogenous VE-cad –
Endogenous VE-cad –
Cleaved VE-cad (N-term) –

Exogenous VE-cad –
Uncharacterized bands

Cleaved VE-cad (C-term) –

Actin –

Vector (–)
Wild
67Δ5
68Δ5
69Δ5

IB: VE-cad
IB: GFP
IB: Actin

– 160 kDa
– 135 kDa
– 95 kDa

– 160 kDa

– 65 kDa

– 45 kDa
**Table S1** siRNA used in this study.

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<td>3’-CAGAGUGGAACAACGUGGACCCAUA</td>
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<td>5’-UUCGGCUGAAUGCACAAGAGCAGC</td>
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We used the same m-calpain siRNA in the human and mouse experiments, as the mRNA sequence targeted by the siRNA is completely conserved between the two species.
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<td>m-Calpain</td>
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<td></td>
</tr>
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<td>CD68</td>
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Table S3 Primary antibodies used for immunoblotting.

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<td>Anti-VE-cadherin pAb: H-72 (Santa Cruz)</td>
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<td>Anti-α-spectrin mAb (Millipore)</td>
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<td><strong>Cloning</strong></td>
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<td>Full-length VE-cad</td>
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<td>3’- GCTGTTGTTGCCTGGAGTCTGACTGGCCTGGG</td>
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<td>C-terminus site lacking a stop codon</td>
<td>5’- GTGATCACCTGCTCATTTCTGCGGC</td>
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<td>3’- ATACAGCAGCTCCCTCCCGGGG</td>
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<td><strong>Mutagenesis study</strong></td>
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<tr>
<td>671Δ5</td>
<td>5’- CGGCCGGGCGCGCAGCTGGACG</td>
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<td>3’- GCCGCCGGCCGCGACCTGC</td>
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<td>681Δ5</td>
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<td>3’- CGACCTGCAGGCGGTCCACGTCGGTC</td>
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<tr>
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<td>5’- CGCACGGTGCGAAGGCGGCGACAC</td>
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<td>3’- GCGTCCACGTCTTCCGGTCCCGGTG</td>
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<td>Diet term</td>
<td>Body weight (g)</td>
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<td>28.0±0.3</td>
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<tr>
<td>HCD</td>
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<tr>
<td><strong>Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</strong></td>
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<td><strong>Inhibitor experiments</strong></td>
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<td><strong>Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</strong></td>
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<tr>
<td>DMSO</td>
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<td>22.3±0.7</td>
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<td>Calpeptin (5 mg/kg/3days)</td>
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<td>ALLM (5 mg/kg/3 days)</td>
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<td>Calpeptin (5 mg/kg/3days)</td>
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<td><strong>In vivo siRNA experiments</strong></td>
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<tr>
<td><strong>Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</strong></td>
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<sup>1</sup>Out of range (<2 mg/dL)

<sup>2</sup>Not detected (invalid data)