m-Calpain antagonizes RhoA overactivation and endothelial barrier dysfunction under disturbed shear conditions

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Aims
It has been reported that laminar shear flow (LF) improves barrier functions in vascular endothelial cells (ECs), whereas disturbed flow (DF) impairs the barrier. Our previous study showed that LF stimulus led to the activation of the cysteine protease, m-calpain, in ECs, which can influence RhoA activity. We hypothesized that m-calpain participates in the shear pattern-dependent EC barrier maintenance through RhoA signalling.

Methods and results
m-Calpain expression levels in the intima in the inferior aspect of mouse aortic arch where DF dominates were higher than those in adjacent regions. Elevation in transendothelial albumin permeability, which was induced by administration of a calpain inhibitor (ALLM), was prominent in the inferior arch; moreover, this elevation was abolished by Rho kinase (ROCK) inhibitor (Y-27632). Similarly, short interfering RNA (siRNA)-induced silencing of m-calpain resulted in increased RhoA activity and hyperpermeability in the aortic arch, which was accompanied by ROCK inhibitor-sensitive phosphorylation of downstream effector LIM kinase 2 (LIMK2), stress fibre accumulation in endothelium and enhanced interendothelial gaps. Exposure of human umbilical vein endothelial cells to LF diminished RhoA activity; in contrast, DF facilitated the activity. siRNA-induced m-calpain silencing further accelerated the DF-induced RhoA overactivation, phosphorylation of LIMK2, and cytoskeletal rearrangement, resulting in barrier dysfunction in the cells.

Conclusion
Our findings revealed relatively high m-calpain expression levels in the inferior arch. The m-calpain activity antagonizes DF-induced overactivation of RhoA/ROCK/LIMK2 signalling and subsequent cytoskeletal rearrangement in ECs, which leads to barrier improvement.

Keywords
Disturbed shear flow • m-Calpain • RhoA • Mouse • Aortic arch

1. Introduction
Endothelial cells (ECs) are known to sense and respond to haemodynamic shear stress, the tangential dragging force generated by blood flow.1 Furthermore, it has been documented that atherosclerotic lesions preferentially occur in vascular regions where disturbed blood flow is dominant (e.g. aortic arch, coronary artery, and aortic bifurcation).2 High atherosclerosis susceptibility in these disturbed shear flow (DF) regions is considered to be, at least in part, due to the limited EC mechanosignalling, as an artificial disruption of laminar shear flow (LF) reportedly accelerates atherogenesis in the straight vessels.3

It has been reported that decreasing EC barrier function is one of the initial symptom in atherogenesis.4 EC barrier regulation is, in general, thought to be accomplished by the coordinated release and reattachment of adhesion molecule complex (e.g. VE-cadherin complex) with the dynamically changing actin cytoskeleton.5 This cytoskeletal dynamics is controlled through Rho GTPase signalling (e.g. RhoA and Rac1 signalling), in some instances.5–7 Seebach et al.8 recently noted that up-regulation of transendothelial electrical resistance as well as junctional recruitment of actin filaments and linearization of VE-cadherin during EC adaptation to LF is dependent on Rac1 activity. Furthermore, several lines of evidence suggested that LF modifies RhoA activity in ECs;9,10 therefore, it is
considered that Rho GTPase plays a functional role in the barrier maintenance during the EC adaptation to LF. On the other hand, Miao et al. previously reported that continuous exposure of cultured ECs to LF stimulus results in intercellent cell–cell junctions and formation of intercellular gaps; in contrast, LF leads to integrated intercellular junctions. Thus, DF stimulus is considered to exert an inhibitory effect on the EC barrier; however, the regulatory mechanisms underlying the DF-induced EC barrier maintenance remain unclear.

In addition to Rho GTPase signalling, calpain, a Ca\(^{2+}\)-dependent intracellular cysteine protease, is believed to influence cell dynamics. Calpain consists of two ubiquitous isoforms, \(\mu\) and \(\mu\)-calpain, which require micromolar and millimolar levels of Ca\(^{2+}\) for half-maximal activation, respectively.\(^{12}\) Our previous investigation indicated that exposure of cultured ECs to DF stimulus results in the activation of \(\mu\)-calpain without altering \(\mu\)-calpain activity.\(^{13}\) Interestingly, Kulkarni et al. documented direct cleavage of RhoA by \(\mu\)-calpain, resulting in RhoA inactivation. This proteolysis appears to influence RhoA-mediated adhesion signalling during EC attachment to its substratum.\(^{14}\) Scalia et al.\(^{15}\) recently documented that the diabetes-induced increases in albumin leakage in mesenteric microcirculation are significantly restored by an antisense depletion of leakage in mesenteric microcirculation are significantly restored.

The investigation conforms with the animal care guidelines and was approved by the Institutional Animal Care and Use Committee of Showa University. Male ddY mice (body weight: 23–30 g) were housed in wire cages, fed standard laboratory chow and allowed access to water ad libitum.

2.3 Real-time reverse transcriptase–polymerase chain reaction

Intima in the superior/inferior aspects of the aortic arch and the proximal/distal thoracic aorta, which was isolated by gentle scalping with a coverslip, was collected employing confocal microscopy (LSM510; Carl Zeiss), and actin filament in endothelium in inferior arch segments was immunohistochemically detected using anti-phospho LIMK2 mAb (Abcam). Actin filament in endothelium in inferior arch segments was stained with Texas Red-labelled phalloidin. One hour after fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA) injection (1.0 mg/body), the inferior arch was excised. In order to visualize interendothelial junctions in the aorta segment, silver nitrate staining was conducted as previously described.\(^{13}\) Series of Z-slice images of the aortic arch and formation of intercellular gaps; in contrast, LF leads to integrated intercellular junctions. Thus, DF stimulus is considered to exert an inhibitory effect on the EC barrier; however, the regulatory mechanisms underlying the DF-induced EC barrier maintenance remain unclear.

The investigation conforms with the animal care guidelines and was approved by the Institutional Animal Care and Use Committee of Showa University. Male ddY mice (body weight: 23–30 g) were housed in wire cages, fed standard laboratory chow and allowed access to water ad libitum.

2.4 In vivo permeability assay

Male ddY mice were anaesthetized, after which they were administered drugs or siRNA via the tail vein. Evans blue dye (EBD) was then injected via the tail vein (1.4 mg/body). Following perfusion fixation with pfa, the aorta segment was carefully harvested (see Supplementary material online, Figure S1) and lysed in 1 M KOH solution. Eluted dye was extracted with extraction buffer (1:13 mixture of 0.2 M H\(_3\)PO\(_4\) and acetonitrile). Following centrifugation, optical absorbance of the extraction buffer was monitored at 595 nm utilizing a microplate reader.

2.5 RNA interference

For in vivo experiments, mice were briefly anaesthetized with diethyl ether, followed by transfection with a small interfering RNA (siRNA) against m-calpain (3 \(\mu\)g/body) or non-silencing control RNA (3 \(\mu\)g/body) involving TransIT-QR Hydrodynamic delivery solution (Mirus Bio) in accordance with the manufacturer’s instructions. The mice were subjected to experiments 48 h following administration. For in vitro experiments, HUVECs were transfected with \(\mu\)-calpain siRNA, \(\mu\)-calpain siRNA, or control RNA involving the siPORT NeoFX Transfection Agent (Ambion) in accordance with the manufacturer’s instructions. Cells were subjected to experiments 48 h following transfection. The nucleotide sequences and efficacy of the siRNA are displayed in Table 1.

2.6 Microscopic analysis

One hour after fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA) injection (1.0 mg/body), the inferior arch was excised. In order to visualize interendothelial junctions in the aorta segment, silver nitrate staining was conducted as previously described.\(^{18}\) Phosphorylated LIM kinase 2 (LIMK2) in endothelium in inferior arch segments was immunohistochemically detected using anti-phospho LIMK2 mAb (Abcam). Actin filament in endothelium in inferior arch segments was stained with Texas Red-labelled phalloidin (Invitrogen). Series of Z-slice images of the aortic intima were collected employing confocal microscopy (LSM510; Carl Zeiss), and were appropriately reconstructed in 3D image.

2.7 Cell culture

HUVECs, which were purchased from Cambrex Inc., were cultured as previously described.\(^{13}\) Confluent cells from 2 to 7 passages were utilized in the experiments.
2.8 Flow experiments

For LF stimulus, the cells were mounted in a conventional parallel flow chamber as previously described. Calculated values for shear stress are 10 dyne/cm², which occur within physiological range. For DF stimulus, the cells were mounted in a modified parallel flow chamber equipped with rectangular steps in the flow path as described previously by Phelps et al. with slight modification (see Supplementary material online, Figures S2 and S3). Flow characteristics in the chamber were determined in Concentration Heat and Momentum Limited Japan by using PHOENICS software (Concentration, Heat chamber were determined in Concentration Heat and Momentum software). On the basis of this characterization, steps were placed perpendicular to the flow path in a 5 mm-interval, in order to effectively subject the cells to DF. In any case, the cells placed in the flow path were perfused with modified Krebs–HEPES buffer (KHB) at 37°C.

2.9 In vitro permeability assay

For measuring EC permeability in vitro, cells cultured on collagen-coated polycarbonate (pour size: 0.4 μm) place on a 1.5% agarose gel (1 mm thickness) were integrated in the DF chamber (see Supplementary material online, Figure S2), as described previously by Phelps et al.21 with slight modification. Subsequent to DF stimulus, flow path was filled with PBS containing 10% BSA and 1 mg/mL EBD, and was then incubated for 30 min in room temperature. Following washing three times, gels were photographed and gel area within 0–3 mm downstream of the flow steps was precisely dissected by using tissue chopper. Dissected gels were lysed in warmed PBS and optical absorbance of the eluted dye was monitored at 595 nm utilizing a microplate reader.

2.10 Immunoblotting

Protein expression levels were determined by immunoblotting as previously described. Primary antibodies used here are displayed in supplementary material online, Table S2.

2.11 RhoA pull-down assay

Whole cell lysate was incubated with GST-RBD bound to agarose beads (20 μg per sample; 45 min) at 4°C. Affinity-precipitated RhoA was separated by PAGE and detected by immunoblotting.

2.12 Overexpression study

cDNA clone of full-length human m-calpain (MGC29448, GenBank accession no. BC021303) was purchased from Invitrogen, and was inserted into restriction site of pcDNA3.1(−) expression vector. Constructed vector (1 μg of DNA) was transiently transfected into HUVECs involving Lipofectamine LTX (Invitrogen) according to manufacturer’s instruction. Cells were subjected to experiments 24 h following transfection.

2.13 Data analysis

Results are expressed as the mean ± SEM. The Mann–Whitney U test was employed to determine the statistical difference between two groups. Multiple comparisons were conducted with analysis of variance (ANOVA), followed by Dunnett’s test. P < 0.05 was considered statistically significant in this study.

3. Results

3.1 Shear pattern-dependent expression of m-calpain in ECs

m-Calpain mRNA expression levels in the intima in the superior/inferior aspect of the murine aortic arch and proximal/distal thoracic aorta were compared (Figure 1A). Real-time RT–PCR analysis indicated that the mRNA level of m-calpain in the inferior arch is relatively high in comparison to that in the superior arch and proximal/distal thoracic aorta. Immunohistochemical analysis similarly revealed relatively high protein expression levels of m-calpain in the intima of inferior arch (Figure 1B).

The effects of flow pattern on calpain expression levels were confirmed in HUVECs by utilizing conventional and modified flow chamber (Figure 1C). Immunoblot analysis revealed that m-calpain protein expression levels in the cells are sustained within 1 h following onset of LF; however, those levels declined significantly at 24 h following LF onset. In contrast, DF did not influence m-calpain expression levels during these periods. m-Calpain expression levels were unchanged by LF or DF stimulus.

3.2 m-Calpain-mediated improvement of EC barrier functions in the inferior arch

To address the roles of calpain in vascular permeability, the effects of pharmacological inhibitors on albumin permeability in the thoracic aorta and aortic arch were investigated employing the EBD permeability assay (Figure 2). In control segments, faint EBD staining was observed at the inferior aspect of the aortic arch (Figure 2A). Administration of ALLM, a pharmacological inhibitor of calpain, in mice induced abnormal focal accumulation of EBD staining, in particular, at the inferior arch. Cross-sectional analysis revealed that EBD staining in the ALLM-treated segment was localized to the luminal side of the vessels (Figure 2B). In contrast, administration of lipopolysaccharides (LPS), a potent inflammatory agent, led to diffuse EBD staining at the aortic arch and thoracic aorta. Quantification suggested that the amount of leaked EBD in the ALLM-treated segments was reliably increased (Figure 2C), which was nearly equal to that in the LPS-treated segment.
Figure 1 Site-specific and shear pattern-dependent m-calpain expression in ECs. (A) Different expression levels of m-calpain mRNA in mouse aorta. Endothelium in the superior/inferior aspect of murine aortic arch and proximal/distal thoracic aorta were isolated as illustrated in the left image. mRNA levels, which were normalized by β-actin expression, are represented as the relative changes against those in the inferior arch (n = 5, *P < 0.05). (B) m-Calpain protein distribution in endothelium in aorta. Series of Z-slice images of the aortic intima were reconstructed in 3D image. Results are representative of four independent experiments. (C) Effects of shear pattern on m-calpain protein expression levels in HUVECs. Cells were exposed either to static culture or to shear stress at 10 dyne/cm². The densitometric amount of the blots, which was normalized by β-actin expression, is presented (n = 4, *P < 0.05 vs. static control).
segments. ALLM-induced EBD staining declined to baseline levels following the administration of Y-27632, an inhibitor of Rho-associated coiled-coil kinase (ROCK),22 in concert with ALLM, whereas the administration of Y-27632 alone did not influence baseline permeability.

The effects of m-calpain silencing on vascular permeability were examined using m-calpain-specific siRNA (Figure 3). Immunoblot analysis showed that m-calpain expression levels in aorta in siRNA-treated mice are significantly diminished by 63.5% in comparison to those in control RNA-transfected mice with no alteration of μ-calpain or β-actin expression (Figure 3A). In addition, immunohistochemical analysis revealed that the m-calpain localization in endothelium in the inferior arch is vanished by the administration of siRNA (Figure 3B). No significant differences in mean arterial blood pressure, heart rate, and aortic blood flow were observed between control and siRNA mice (see Supplementary material online, Table S3). The EBD/BSA assay was then performed in aortic arch, lung, and kidney in control and m-calpain knockdown mice (Figure 3C). As a result, EBD/BSA leakage in the aortic arch segments, but not in the other organs examined, was promoted by the siRNA administration. Furthermore, the integrity of the interendothelial junction was evaluated with silver nitrate staining (Figure 3D). Even in control mice, intercellular gaps were detected along the junctional region of ECs in the inferior arch. Both density and mean size of the gaps in hyperpermeable regions in the inferior arch of siRNA-treated mice were remarkably increased relative to

**Figure 2** Vascular hyperpermeability induced by the calpain inhibitor in the inferior arch. Mice received ALLM (0.1 or 1.0 mg/kg), LPS (1 mg/kg), or Y-27632 (0.1 mg/kg). One hour following administration, EBD was injected via the tail vein (1.4 mg/body). (A) Photographs indicate the en-face distribution of EBD in the three representative aorta segments isolated from control, ALLM (1.0 mg/kg)-, ALLM (1.0 mg/kg)/Y-27632-, or LPS-treated mice. Scale bars indicate 2 mm. Arrows represent dye leakage. (B) Cross-sectional image of the ALLM-treated segment. Scale bars indicate 200 μm. Arrows represent dye leakage. (C) Quantification of leaked dye in the segments. Values, which were normalized by wet weight of the organs, are presented (n = 4–5, *P < 0.05 vs. vehicle, #P < 0.05 vs. ALLM alone).
Figure 3  Vascular hyperpermeability induced by m-calpain silencing in the inferior arch. Mice were hydrodynamically transfected with control RNA (3 μg/body) or siRNA against m-calpain (3 μg/body; sequence 1). (A) Selective silencing of m-calpain. Protein expression levels in whole aortic arch segments were analysed by immunoblotting. The densitometric amount of the blots, which was normalized by β-actin expression, is presented (n = 4, *P < 0.05 vs. control). (B) m-Calpain protein distribution in endothelium in inferior arch. Results are representative of four independent experiments. Scale bars indicate 400 μm. (C) Hyperpermeability induced by m-calpain silencing in aortic arch. EBD was injected via the tail vein (1.4 mg/body). LPS (1 mg/kg) was administrated to mice as a positive control. The amount of the leaked dye, which was normalized by wet weight of the organs, is presented (n = 4, *P < 0.05 vs. control). (D) Formation of interendothelial gaps induced by m-calpain silencing in inferior arch and thoracic aorta. Interendothelial junctions were visualized with silver nitrate staining. Arrows represent interendothelial gaps. Distributions of leaked FITC-BSA in the corresponding fields are indicated in the insets. Results are representative of four segments derived from individual animals. Scale bars indicate 20 μm.
those of control mice (see Supplementary material online, Table S4); in contrast, few gaps were observed in the thoracic aorta segments despite siRNA treatment.

3.3 m-Calpain-mediated down-regulation of RhoA/cytoskeletal signalling in ECs in inferior arch

Control and siRNA-treated vessels were subjected to pull-down assay in terms of RhoA activity (Figure 4A). Accordingly, RhoA activity in siRNA-treated segments was facilitated relative to that in control segments, whereas RhoA protein expression levels were similar between the segments. The relative amount of calpain-generated dominant negative form of RhoA (≈19 kDa) was unaffected by m-calpain silencing (Figure 4A). Subsequently, phosphorylation of LIMK2, a downstream target of RhoA, was immunohistochemically analysed in ECs (Figure 4B). In control animals, phospho-LIMK2-positive ECs were rarely observed in the intima of inferior arch. An administration of siRNA dramatically accumulated the positive cells in this region (see Supplementary material online, Figure S4). This LIMK2 phosphorylation was remarkably inhibited by the administration of Y-27632. LIMK2 phosphorylation levels in ECs in the thoracic aorta were lesser than that in the aortic arch, and were unchanged despite siRNA treatment.

In addition, cytoskeletal distribution in endothelium in the inferior arch in control and siRNA-treated mice was investigated (Figure 4C). In control mice, the junctional recruitment of actin filament was observed in ECs in the inferior arch. ECs in the hyper-permeable regions in the inferior arch of m-calpain knockdown mice displayed cytoskeletal rearrangement, which is characterized by declining expression of junction-associated actin filaments and enhanced formation of stress fibre in the central region of the cells. This cytoskeletal rearrangement was resembled with that induced by LPS administration, and was impaired in the presence of Y-27632. In contrast to the arch, cytoskeletal distribution in ECs in the thoracic aorta of m-calpain knockdown mice was unchanged in comparison with that of control mice (see Supplementary material online, Figure S5).

3.4 m-Calpain-mediated inhibition of RhoA overactivation, cytoskeletal rearrangement, and barrier dysfunction in ECs in response to DF

RhoA activity was tested in HUVECs under the conditions of LF or DF (Figure 5A). Pull-down assay indicated that RhoA activity decreases within 3 min following initiation of LF stimulus. Furthermore, LF-induced RhoA down-regulation was sustained for at least 60 min following shear onset, whereas RhoA protein expression levels in the cells were not altered during this period. In contrast to LF, DF stimulus strongly facilitated the activity, which was sustained for at least 60 min following shear onset. The relative amount of calpain-generated dominant negative form of RhoA (≈19 kDa) was unaffected by the LF or DF stimulus (Figure 5A).

Subsequently, we examined the effects of m-calpain silencing on RhoA activity in HUVECs under the LF and DF conditions (Figure 5B). m-Calpain expression levels in siRNA-treated cells were diminished by 61.1% (Table 1). The siRNA treatment prevented the LF-induced RhoA down-regulation, and facilitated DF-induced RhoA overactivation.

Dependence of m-calpain expression levels on DF-induced phosphorylation of LIMK2 was evaluated by immunoblotting using phospho-specific antibody. LIMK2 phosphorylation in control RNA-transfected cells was elevated by the DF stimulus in comparison with that in the cells subjected to static culture (Figure 5C). DF-induced LIMK2 phosphorylation was further accelerated by m-calpain silencing, but not by μ-calpain silencing. Furthermore, an alternative m-calpain siRNA showed a similar facilitatory effect (see Supplementary material online, Figure S6). In contrast, an overexpression of m-calpain significantly attenuated the DF-induced LIMK2 phosphorylation (Figure 5D).

The effects of shear pattern on proteolysis of focal adhesion molecule vinculin were tested (Figure 5E). As a result, vinculin proteolysis was observed in HUVECs both under the DF and LF conditions. DF-induced vinculin proteolysis was relatively weak comparing with that induced by LF stimulus.

Roles of m-calpain in DF-induced barrier dysfunction were investigated in HUVECs. In vitro EBD assay showed no remarkable dye leakage in the control cells in static culture. The control cells subjected to DF stimulus displayed an increasing permeability in particular in the downstream region of the flow step (Figure 6A). Quantification showed that m-calpain silencing further accelerates dye leakage in the downstream region (Figure 6B). This elevation in dye leakage was suppressed by Y-27632.

Shear pattern-dependent roles of m-calpain in LIMK2 phosphorylation and cytoskeletal dynamics in HUVECs were examined (Figure 6C). Computational fluid dynamics analysis (see Supplementary material online, Figure S3) showed that recirculation flow, separated flow, and LF are found in region I, II, and III in the DF chamber, respectively. Immunocytochemical analysis revealed that exposure of the control cells to LF stimulus leads to accumulation of actin filaments along the junctional region (region III in upper images in Figure 6C), while the cells in static culture showed less organized actin cytoskeleton (see Supplementary material online, Figure S7). The cells subjected to recirculation and separated flow did not display the junctional accumulation of actin filaments (region I and II in upper images in Figure 6C, respectively). Phospho-LIMK2 in these control cells did not spatially correspond with actin cytoskeleton. In contrast, m-calpain knockdown cells did not exhibit junctional accumulation of actin filaments under the LF conditions (region III in lower images in Figure 6C). Alternatively, exposure of m-calpain knockdown cells to recirculation and separated flow resulted in robust stress fibre formation (region I and II in lower images in Figure 6C, respectively). The separated flow stimulus, in addition, elicited significant co-localization of phospho-LIMK2 and actin cytoskeleton in junctional and cytoplasmic regions in m-calpain knockdown cells.

4. Discussion

In the present study, an administration of ALLM or m-calpain siRNA to mice accelerated transendothelial permeability, preferentially at the inferior arch where DF dominates (Figures 2 and 3). Similar to in vivo experiments, m-calpain silencing facilitated
Figure 4 RhoA overactivation and cytoskeletal rearrangement in endothelium induced by m-calpain silencing. Control RNA (3 μg/body), siRNA against m-calpain (3 μg/body; sequence 1), Y-27632 (0.1 mg/kg), or LPS (1 mg/kg) was intravenously administrated to the mice. (A) RhoA overactivation induced by m-calpain silencing in aortic arch. The whole aortic arch segments were analysed. The densitometric amount of GTP-bound RhoA, which was normalized by total RhoA expression levels, is indicated in left graph (n = 4, *P < 0.05 vs. control). RhoA proteolysis (ratio of 19 to 23 kDa fragment) is presented in right graph (n = 4, *P < 0.05 vs. control). (B) LIMK2 phosphorylation induced by m-calpain silencing in endothelium in inferior arch and proximal thoracic aorta. The density of the phospho-LIMK2-positive ECs was estimated from the immunohistochemical images of phospho-LIMK2 (see Supplementary material online, Figure S4). Three independent fields (0.30 mm²) were evaluated per specimen (n = 4, *P < 0.05 vs. control). (C) Rearrangement of F-actin cytoskeleton induced by m-calpain silencing in endothelium in inferior arch. Distributions of leaked FITC-BSA in the corresponding fields are indicated in the insets. Results are representative of four segments derived from individual animals. Scale bars indicate 20 μm.
Figure 5  m-Calpain-mediated modification of RhoA/LIMK2 signalling in HUVECs under the LF and DF conditions. Cells in the LF or DF chamber were exposed either to static culture or to shear stress at 10 dyne/cm². (A) Temporal changes in LF- and DF-induced RhoA activity in HUVECs. Upper and lower graphs indicate RhoA activity and RhoA proteolysis (ratio of 19 to 23 kDa fragment), respectively (n = 4, *p < 0.05 vs. 0 min). (B) Effects of m-calpain silencing on RhoA activity. The cells transfected with control or m-calpain siRNA (sequence 1) were exposed to shear stress for 60 min (n = 3–4, *p < 0.05 vs. static control). (C) Effects of m-calpain silencing on DF-induced LIMK2 phosphorylation. The cells transfected with control, m-calpain, or m-calpain siRNA (sequence 2) were exposed to DF for 60 min. Values, which were normalized by amounts of total LIMK2, are presented (n = 4, *p < 0.05 vs. static control). (D) Effects of m-calpain overexpression on DF-induced LIMK2 phosphorylation. The cells transfected with empty vector or that containing human m-calpain gene (mCAPN OE) were exposed to DF for 60 min (n = 4, *p < 0.05 vs. static control). (E) Effects of LF and DF on vinculin proteolysis in HUVECs. Cells were exposed to shear stress for 60 min. Lower graph indicates ratio of calpain-generated fragment (95 kDa) to 120 kDa intact fragment (n = 3, *p < 0.05 vs. static).
**Figure 6** Effects of m-calpain silencing on DF-induced barrier dysfunction and cytoskeletal dynamics in HUVECs. The cells transfected with control or m-calpain siRNA (sequence 1) in the DF chamber were exposed either to static culture or to shear stress at 10 dyne/cm² for 60 min. In the Y-27632 experiments, cells were pretreated with Y-27632 at 10 μM for 20 min; subsequently, cells were sheared with KHB containing 10 μM Y-27632. (A) Effects of m-calpain silencing on DF-induced barrier dysfunction. Left representative gel images indicate distribution of EBD/BSA that leaked across the HUVEC monolayer. Dotted line in the images represents flow step. Scale bars indicate 2 mm. Right graph indicates densitometric analysis of the gel images. (B) Quantification of leaked EBD/BSA. Gels in 0–3 mm downstream of the steps were dissected and leaked EBD/BSA containing in the dissected gels were quantified (n = 4–5, *p < 0.05 vs. static control). (C) Subcellular localization of F-actin and phospho-LIMK2 in HUVECs under the recirculation, separated flow and LF conditions. Cells were transfected with ALEXA555-labelled control RNA or siRNA against m-calpain. Immunofluorescence images obtained at each observation field-to-step distance (region I: 0–0.2 mm, region II: 0.2–0.6 mm, region III: 4.5–5.0 mm) are illustrated as overlaying images. Arrows represent overlay of actin and phospho-LIMK2 staining. Results are representative of four independent experiments. Scale bars indicate 20 μm.
barrier dysfunction in DF-stimulated HUVECs (Figure 6). It is noteworthy that ALLM preferentially inhibits m-calpain; Ki value of ALLM to m-calpain was two-fold lower than those to \( \mu \)-calpain.\(^9\) Interestingly, our current results demonstrated that the hyperpermeable regions in aorta are spatially corresponded with m-calpain-enriched regions (Figure 1). In vitro experiments suggested that m-calpain expression in ECs is down-regulated by the prolonged application of LF, but not of DF (Figure 1), probably resulting in relatively high expression levels of m-calpain in the inferior arch in comparison with those in adjacent regions. Collectively, it is thought that the EC barrier integrity in the inferior arch is ensured potentially by shear pattern-dependent m-calpain activity.

It has been previously documented that Rho GTPase-mediated modification of junctional actin dynamics, as well as stress fibre formation, is closely related to maintaining EC barrier.\(^23\) Current in vivo and in vitro experiments demonstrated that EC hyper-permeability induced by calpain inhibition is abolished following administration of Y-27632 (Figures 2 and 6). Considering that m-calpain silencing resulted in enhanced RhoA activity and Y-27632-sensitive stress fibre formation in endothelium in aortic arch (Figure 4), m-calpain is thought to potentially improve EC barrier functions in inferior arch by down-regulating RhoA signalling and subsequent cytoskeletal rearrangement.

On the other hand, these in vitro experiments indicated that RhoA activity is dependent on the shear stress-pattern (Figure 5). It is noteworthy that the LF-induced RhoA down-regulation is resembled with that reported in the previous investigation.\(^9\) Current siRNA experiment further demonstrated that m-calpain potentially down-regulates the RhoA activity in ECs both under the LF and DF conditions (Figure 5). Although \( \mu \)-calpain reportedly proteolyses RhoA, which directly inactivates it,\(^14\) current results indicated that m-calpain-induced activity does not significantly contribute to RhoA proteolysis (Figures 4 and 5). Thus, shear stress-induced m-calpain activity may indirectly modulate Rho signalling.

It has been reported that LIMK2 is associated with actin cytoskeletal dynamics through phosphorylative inactivation of actin binding protein cofilin.\(^24\) Garcia et al.\(^7\) documented that sphingosine 1-phosphate-induced accumulation of junctional actin filaments and increases in transendothelial electrical resistance are significantly attenuated by overexpression of exogenous cofilin, suggesting that barrier maintenance in ECs is cofilin-dependent. Interestingly, it appears that LIMK2 activity is functionally coupled with shear stress-induced RhoA/ROCK signalling.\(^25\) Current in vivo and in vitro data suggested that m-calpain expression levels negatively correlate with LIMK2 phosphorylation levels (Figures 4 and 5). Furthermore, m-calpain silencing in DF-stimulated cells led to co-localization of phospho-LIMK2 and stress fibres (Figure 6). These observations are consistent with our current findings that m-calpain activity interferes with DF-induced RhoA signalling and subsequent cytoskeletal rearrangement.

It is known that focal adhesion dynamics, as well as intercellular adhesion dynamics, influences EC barrier functions.\(^13\) We have previously documented that m-calpain proteolyses focal adhesion molecules vinculin and talin in sheared HUVECs, thereby direct modification of focal adhesion dynamics.\(^13\) Current results indicated that the DF stimulus elicits similar vinculin proteolysis; however the DF-induced proteolysis was much less than that induced by LF (Figure 5). Considering that Y-27632 largely restores EC barrier dysfunction induced by calpain inhibition (Figures 2 and 6), it is believed that m-calpain improves EC barrier integrity under the DF conditions primary by attenuating Rho-mediated signalling.

In conclusion, this study demonstrated, for the first time, that m-calpain localizes in the inferior arch where DF dominates, and moreover, that m-calpain down-regulates DF-induced RhoA/cytoskeletal signalling, which exerts subsequent improvement of EC barrier functions. We believe that this down-regulation of RhoA signalling underlies an important implication for atheroprotection, since ROCK reportedly contributes to early atherosclerotic lesion formation.\(^26\) Future investigations are necessary to identify m-calpain behaviour in blood vessels in atherogenic animal models.

### Supplementary material

Supplementary material is available at Cardiovascular Research online.

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### Conflict of interest

none declared.

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