Requirement of Ca\(^{2+}\) influx- and phosphatidylinositol 3-kinase-mediated m-calpain activity for shear stress-induced endothelial cell polarity

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Miyazaki T, Honda K, Ohata H. Requirement of Ca\(^{2+}\) influx- and phosphatidylinositol 3-kinase-mediated m-calpain activity for shear stress-induced endothelial cell polarity. Am J Physiol Cell Physiol 293: C1216–C1225, 2007. First published June 27, 2007; doi:10.1152/ajpcell.00083.2007.—Proteolytic activity in sheared human umbilical vein endothelial cells (HUVECs) was measured using a fluorescent substrate and laser scanning confocal microscopy to clarify the key role of an intracellular Ca\(^{2+}\)-sensitive protease, calpain, in these cells in response to shear stress. Within physiological shear range, activity in the cells was enhanced in shear-dependent fashion. Short interfering RNA-induced silencing of m-calpain, but not of \(\mu\)-calpain, suppressed the activity. Either removal of extracellular Ca\(^{2+}\) or application of an intracellular Ca\(^{2+}\) chelator (BAPTA/AM) or nonselective cation channel blocker (Gd\(^{3+}\)) reduced proteolytic activity. Furthermore, activity was suppressed by phosphatidylinositol bisphosphate (PIP\(_2\)) chelator (neomycin) or phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002); in contrast, activity, which was partially inhibited by ERK kinase inhibitor (U0126, PD98059), was unaffected by PLC inhibitor (U73122). Moreover, Akt phosphorylation downstream of PI3K, which was elicited by shear, was attenuated by neomycin but not by calpain inhibitor (calpeptin). Following assessment of shear-stimulated focal adhesion (FA) and cytoskeletal dynamics using interference reflection/green fluorescence protein-actin microscopy, we found that either calpain or PI3K inhibition impaired shear stress-induced polarization of FAs via stabilization of FA structures. Additionally, HUVEC alignment and cytoskeletal remodeling, which was accompanied by calpain-mediated cleavage of vinculin and talin, were also elicited by prolonged application of shear and impaired by m-calpain knockdown. Thus, these results revealed that physiological shear stress elicits Ca\(^{2+}\) influx-sensitive activation of m-calpain in HUVECs. This activity is facilitated primarily through the PI3K pathway; furthermore, it is essential for subsequent FA reorganization and cell alignment under shear conditions.

mechanotransduction; morphological change; focal adhesion reorganization; fluid flow

BLOOD FLOW GENERATES VARIOUS types of hemodynamic forces—hydrostatic pressure, cyclic strains, and wall shear stresses, which play a pivotal role in physiological and pathophysiological regulation of vascular function. It is well known that shear stress, which is directly exposed to vascular endothelial cells (ECs), modulates EC functions by eliciting mechanotransduction, which includes production of biochemical mediators and motility responses (8). In particular, shear stress-induced regulation of EC motility is of prominent importance in vascular integrity as the dynamics of the cells is directly associated with vascular remodeling (26), as well as with pathogenesis of atherosclerosis (7) and EC alignment in the direction of the long axis of blood vessels (8).

Spontaneously occurring morphological changes in cultured subconfluent ECs exhibit a random-walk pattern; moreover, EC motility patterns are altered under the shear conditions (25). Changes are characterized by the development of leading edges (lamellipodia) and retraction of rear edges in the direction of flow. Lamellipod development and rear retraction appear to be regulated mainly through Rac- and Rho-mediated signaling, respectively (26). Additionally, phosphatidylinositol 3-kinase (PI3K) and ERK in ECs, which are simultaneously activated by shear stimulus (15), are believed necessary for shear stress-induced EC motility (26, 36). On the other hand, several lines of evidence suggested that intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) mobilization is elicited upon application of shear stress in ECs (21, 22, 28, 38); thus, exact modulation of EC motility under shear conditions on the basis of [Ca\(^{2+}\)]\(_i\) changes, in addition to the pathways described above, is expected. Indeed, shear stress-induced alignment of confluent ECs is reportedly eliminated by chelation of intracellular Ca\(^{2+}\) using quin-2/AM (27). However, details regarding [Ca\(^{2+}\)]\(_i\)-mediated regulation of EC motility in response to shear stimulus remain sketchy.

Several lines of evidence revealed that a Ca\(^{2+}\)-dependent intracellular cysteine protease, calpain, which is localized to FA sites (3), implicates cell motility through cleavage of FA components, including the integrin \(\beta 3\) subunit (10), as well as talin (3), focal adhesion kinase (FAK) (6), and vinculin (33). The relationship between calpain activity and cell motility is well documented in EGF-stimulated fibroblast cells (18, 19, 34). It is noteworthy that [Ca\(^{2+}\)]\(_i\) mobilization is not required for calpain activation in these cells (18). This phenomenon is reportedly due to reduction of the Ca\(^{2+}\) requirement of m-calpain, which is potentiated through the ERK- and phosphatidylinositol bisphosphate (PIP\(_2\))-mediated pathway (18, 34), indicating that calpain activity in living cells can be modified through additional [Ca\(^{2+}\)]\(_i\)-independent mechanisms.

Calpain consists of two ubiquitous isozymes, \(\mu\)- and m-calpain, which require micromolar and millimolar levels of Ca\(^{2+}\) for half-maximal activation, respectively (20). Earlier investigations demonstrated the expression and activity of both \(\mu\)- and m-calpain isozymes in ECs (13, 30). In previous research using immunofluorescence microscopy, Ariyoshi et al. (2) interestingly proposed that at least the immunoreactivity of the autolyzed (activated) form of m-calpain accumulated in the peripheral regions of sheared ECs. More recently, Butcher et al. (4) indicated that treatment of ECs with pan...
calpain inhibitor impaired shear stress-induced cell alignment. These observations demonstrate that calpain may be activated in ECs in response to shear stress and that calpain is probably involved in the modulation of shear stress-induced motility; however, the following points were not evaluated in the current study: 1) the isozyme-selectivity of shear stress-induced calpain activity, 2) the \([\text{Ca}^{2+}]\)-sensitive or insensitive mechanism(s) underlying calpain activation, and 3) the signal transduction pathway involved in the calpain-mediated regulation of EC motility under shear conditions.

We previously demonstrated that fluid flow-induced \([\text{Ca}^{2+}]\) transients in ECs are initiated from peripheral FAs, followed by rear retraction or termination of lamellipod development (28). \([\text{Ca}^{2+}]\) transients and rear retractions are suppressed by Gd\(^{3+}\), a nonselective cation channel blocker (21); as a result, it is hypothesized that shear stress-induced EC motility is controlled by \([\text{Ca}^{2+}]\) transients via calpain-mediated modulation of FA dynamics. To address this hypothesis, intracellular proteolytic activity in living HUVECs was measured in this study with the fluorogenic proteolysis indicator bis(benzyloxycarbonyl-L-alanyl-L-ala-nyl-L-alamine amide)-rhodamine 110 (bCAA-R110), using laser scanning confocal microscopy (LSCM). Consequently, the present findings revealed, for the first time, that application of physiological shear stress causes selective activation of m-calpain in HUVECs, primarily via the PI3K-mediated pathway in a \([\text{Ca}^{2+}]\) influx-dependent manner, although the ERK-mediated pathway plays a role. This activity is necessary for subsequent HUVEC alignment by inducing FA polarization.

**MATERIALS AND METHODS**

**Reagents.** Calcein red-orange/AM, bCAA-R110 and BAPTA/AM were obtained from Invitrogen. U73122, LY294002, and phallolidin conjugated to tetramethylrhodamine B isothiocyanate (TRITC) were purchased from Sigma. PD98059 and U0126 were obtained from Calbiochem/EMD Biosciences; Gd\(^{3+}\) was acquired from Wako Pure Chemical Industries. Neomycin was obtained from MP Biomedicals. All other chemicals were commercial products of the highest available grade of purity.

**Cell culture.** Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex and were cultured according to the manufacturer’s instructions. HUVECs were cultured in endothelial cell basal medium-2 (EBM-2; Cambrex) containing 2% FBS under humidified conditions (95% air-5% CO\(_2\)) at 37°C. In all cases, the flow systems were maintained at 37°C. The values for shear stress (\(\tau\)) were calculated (22, 38) from the following equation: \(\tau = \rho Q/hw^2\), where \(h\) and \(w\) represent the height and width of the flow path, respectively, \(Q\) denotes the flow rate and \(\rho\) is the viscosity of KHB (0.76 mPa s).

**Proteolytic activity measurement.** Proteolytic activity was measured using a fluorogenic membrane-permeable substrate (bCAA-R110), according to the procedure proposed by Gitler and Spira (16) with slight modification. In brief, a HUVEC monolayer integrated with siPORT NeoFX Transfection Agent (Ambion). Twenty-four hours after transfection, the flow systems were divided into upstream and downstream regions at the cell’s centroid, which was established with National Institutes of Health (NIH) Image software, for analysis of FA motility (Fig. 6B); subsequently, total FA area per cell area was measured distinctly in the upstream and downstream regions. Cell area was determined using GFP-actin images. To estimate FA area, acquired IRM images were thresholded so as to disclose black FAs on a white background (Figs. 5 and 6). To evaluate FA stability (Fig. 6A), FAs, which were extracted from IRM images acquired before and 50 min following shear onset, were carefully merged to disclose black FAs on a white background. Total area of stable FAs that remained stationary during this period was then measured. Cells were divided into upstream and downstream regions at the cell’s centroid, which was established with National Institutes of Health (NIH) Image software, for analysis of FA motility (Fig. 6B); subsequently, total FA area per cell area was measured distinctly in the upstream and downstream regions. Cell area was determined using GFP-actin images.

**Immunofluorescence.** Cells were fixed in 4% paraformaldehyde, permeabilized with 0.4% Triton X-100, and blocked with 10% TSA. Subsequently, cells were incubated with anti-vinculin Mab (1:400) followed by incubation with FITC-conjugated anti-\(\text{IgG}\) Mab (1:500). Actin filaments were stained with TRITC-conjugated phalloidin (1:500 for 30 min). Fluorescence images were collected using a LSCM (RTS-2000MP; Bio-Rad) equipped with a \(\times 40\) oil immersion objective lens (1.3 NA) and appropriate filter sets.

**Shear stress application.** HUVECs were subjected to shear stress using parallel flow chambers developed in our laboratory (See the online version of this article contains a supplemental for details.). Cells placed in the glass-bottomed flow path (height/width/length: 0.2x1x40 mm) were perfused with KHB using a syringe pump (Muramachi Kikai) for measurement of proteolytic activity (Figs. 1–3) or GFP-actin dynamics (Figs. 5 and 6). Cells placed in the polystyrene-bottom flow path (height/width/length: 0.2x3x120 mm) were perfused with gravity-fed KHB for preparation of lysates for Western blot analysis (Figs. 1B, 4, and 7). In the cell alignment experiment (Fig. 8), cells placed in the glass-bottomed flow path (height/width/length: 0.2x3x40 mm) were perfused with gravity-fed DMEM containing 1% FBS. In all cases, the flow systems were maintained at 37°C. The values for shear stress (\(\tau\)) were calculated (22, 38) from the following equation: \(\tau = \rho Q/hw^2\), where \(h\) and \(w\) represent the height and width of the flow path, respectively, \(Q\) denotes the flow rate and \(\rho\) is the viscosity of KHB (0.76 mPa s).

**RNA interference.** HUVECs were transfected with either a Stealth small interfering RNA against \(\mu\)-calpain (5’-UAGAGUGGUCCUAUUGCGGGA-3’; Invitrogen) or 40 nM, m-calpain (5’-UUCGCGUAAGGCACAAAAGCAGG-3’; Invitrogen) at 40 nM, or nonsilencing control RNA (medium GC; Invitrogen) at 40 nM, involving the siPORT NeoFX Transfection Agent (Ambion), in accordance with the manufacturer’s instructions. Cells were subjected to the experiments 24 h following transfection.

**Immunoblotting.** HUVECs were lysed in ice-cold lysis buffer (20 mM Tris-HCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate; pH 7.4), containing protease inhibitor cocktail (Sigma). A protein aliquot (10 \(\mu\)g) was subjected to electrophoresis on 7.5% polyacrylamide gel containing SDS under reducing conditions; subsequently, proteins in the gel were transferred to a nitrocellulose membrane. The membranes were blocked with 1% casein, after which they were reacted overnight at 4°C with primary antibodies (see Table 1 for details). After being washed three times, membranes were incubated for 2 h at room temperature with horseradish peroxidase-conjugated anti-IgG Mabs. Next, proteins were detected with the ECL reagent (Bio-Rad Laboratories).

**Immunofluorescence.** Cells were fixed in 4% paraformaldehyde, permeabilized with 0.4% Triton X-100, and blocked with 10% BSA. Subsequently, cells were incubated with anti-\(\text{IgG}\) Mab (1:400) followed by incubation with FITC-conjugated anti-\(\text{IgG}\) Mab (1:500). Actin filaments were stained with TRITC-conjugated phalloidin (1:1,000 for 30 min). Fluorescence images were collected using a LSCM (RTS-2000MP; Bio-Rad) equipped with a \(\times 40\) oil immersion objective lens (1.3 NA) and appropriate filter sets.
Data analysis. Images were analyzed with NIH Image and ImageJ software. Paired or unpaired t-test was appropriately used to determine the statistical difference between two groups. Multiple comparisons were conducted with ANOVA, followed by Dunnett’s test. \( P < 0.05 \) was considered statistically significant in this study.

RESULTS

Association of m-calpain with shear stress-induced proteolytic activity in HUVECs. HUVECs were simultaneously loaded with bCAA-R110 and calcein red-orange to measure proteolytic activity (Fig. 1A). Application of shear stress at 30 dyne/cm\(^2\) resulted in sustained increases in R110 fluorescence intensity in the cells, which suggested an enhancement of intracellular proteolytic activity in the cells. These increases peaked at 20.2 \( \pm \) 0.6 min following onset of shear with peak \( \Delta F/F_0 \) values of 1.56 \( \pm \) 0.16 (means \( \pm \) SE, \( n = 3 \)). Conversely, calcein-fluorescence intensity was not affected by shear, indicating that the increases are not due to artifacts such as changes in cellular thickness or incorrect focal plane positioning. A-
activity was impaired by calpeptin, a pharmacological inhibitor of calpain (20), but not by PMSF, a broad-range serine protease inhibitor (35) (See supplemental Fig. 2 in the online version of this article). Indeed, treatment with 1 μM calpeptin reduced shear stress-induced activity by 85% compared with that of control cells. Additionally, both μ- and m-calpain expression levels were not significantly altered under shear conditions within 30 min following shear onset (Fig. 1B).

Subsequently, the effects of gene silencing of calpain isozymes on proteolytic activity were examined. Western blot analysis revealed that transfection of μ-calpain siRNA significantly diminished μ-calpain expression levels by 55.2 ± 12.3% (means ± SE, n = 4) compared with those of control RNA-transfected cells, with no alteration of m-calpain or β-actin expression (Fig. 1C). In contrast, m-calpain levels in cells transfected with m-calpain siRNA were selectively reduced by 51.0 ± 8.2% (means ± SE, n = 4), which indicated the absence of substantial off-target effects. Shear stress-induced proteolytic activity in cells was assessed using bCAA-R110 (Fig. 1D). Activity in μ-calpain-knockdown cells was identical to that in control RNA-transfected cells; on the other hand, m-calpain-knockdown cells did not respond to the stimuli.

In addition, application of the Ca2+ ionophore ionomycin strongly elicited activity in control RNA-transfected cells (See supplemental Fig. 3 in the online version of this article). This activity was equally attenuated in μ- and m-calpain-knockdown cells.

Mechanisms underlying shear stress-induced calpain activation. HUVECs loaded with bCAA-R110 were exposed to different shear stresses to clarify the dependence of proteolytic activity on shear stress (Fig. 2A). In cells subjected to static conditions (0 dyne/cm²), R110 fluorescence intensity remained unchanged during the observation period for 30 min. Increasing shear stress induced elevations in the maximum level of activity, which was apparent at shear stresses up to 10 dyne/cm². Statistically significant differences between the static control and test groups were observed with respect to shear stress up to 30 dyne/cm².

The Ca2⁺ requirement of proteolytic activity was examined (Fig. 2B). Removal of extracellular Ca²⁺ or introduction of Gd³⁺ equally attenuated maximal elevations of activity. Similarly, pretreatment with BAPTA/AM, a cell-permeable Ca²⁺ chelator, suppressed activity.

The effects of PD98059 and U0126, highly selective ERK kinase inhibitors (1, 11), on shear stress-induced proteolytic activity were evaluated (Fig. 3A). Statistically significant reductions in the maximal increases in activity were evident following PD98059 treatment. Note that a considerably higher concentration (30 μM) of inhibitor could not completely eliminate activity. Similar inhibitory effects were observed in U0126-treated cells.

The effects of neomycin, a high-affinity PIP2 scavenger (14), LY294002, a selective PI3K inhibitor (9), and U73122, a broad-range PLC inhibitor (30), were compared in terms of shear stress-induced proteolytic activity (Fig. 3B). Rising concentrations of inhibitor could not completely block shear stress-induced activity in control RNA-transfected cells. In contrast, RNA-transfected cells with m-calpain-knockdown activity were equally attenuated in the presence of either neomycin or LY294002, which strongly elicited activity in control RNA-transfected cells (See supplemental Fig. 3 in the online version of this article).

Table 1. **Primary antibodies used for immunoblotting**

<table>
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<tr>
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<td>Anti-m-calpain antibody (Sigma)</td>
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<td>Anti-talin MAb (Sigma)</td>
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<tr>
<td>Anti-FAK MAb (BD Biosciences)</td>
<td>77</td>
</tr>
<tr>
<td>Anti-phospho-Akt (Ser473) antibody</td>
<td>1:1000</td>
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<td>Anti-Akt antibody (Cell Signaling Technology)</td>
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**Fig. 2.** Shear stress and Ca²⁺ dependency of shear stress-induced calpain activity in HUVECs. Cells loaded with bCAA-R110 were usually exposed to shear stress at 0 dyne/cm² with the exception of the shear stress-dependency experiment. Temporal F/F₀ changes in the cells during the first 30 min following shear onset appear in the line graph. Maximal F/F₀ elevations (ΔF/F₀) during this period are represented in the bar graph. A: shear stress dependency of activity. F/F₀ changes in cells subjected to static conditions or shear stress (3, 10, 30, or 50 dyne/cm²) are represented. Results are expressed as the means ± SE (n = 3). *P < 0.05 compared with 0 dyne/cm²-group. B: Ca²⁺ dependency of activity. To eliminate extracellular Ca²⁺, flow solution was replaced with Ca²⁺-depleted Krebs-HEPES buffer (KHB) [Ca²⁺(−)]. In the Ca²⁺ experiments, cells were pretreated with Gd³⁺ at 3 μM for 20 min; subsequently, cells were sheared with KHB containing 3 μM Gd³⁺ (Gd³⁺). In the BAPTA/AM experiments, cells were pretreated with BAPTA/AM at 50 μM for 20 min, followed by shearing with normal KHB (B/AM). Results are expressed as the means ± SE (n = 4–7). *P < 0.05 compared with control group.
concentrations of neomycin resulted in dose-dependent reductions of the maximal increases in shear stress-induced activity. LY294002 also prevented proteolytic activity. In contrast, U73122 at considerably higher concentrations (1–10 μM) exerted no inhibitory effects; rather, U73122 more likely promoted proteolytic activity.

Additionally, the inhibitory effects of neomycin and U73122 on PLC-mediated proteolytic activity in HUVECs were tested (See supplemental Fig. 4 in the online version of this article). Treatment of cells with ATP, a PLC activator (37), resulted in a detectable, albeit modest, enhancement of proteolytic activity. The increases were attenuated substantially in the presence of U73122 or neomycin, suggesting that U73122 concentrations used in the present investigation were sufficient for inhibition of PLC activity.

The inhibitory effects of LY294002, neomycin, or calpeptin on shear stress-induced phosphorylation of Akt, which is downstream of PI3K, were confirmed (Fig. 4). Sheep stress-induced phosphorylation of Akt in ECs was described previously (9). Similar phosphorylation was observed in sheared HUVECs in the current study (Fig. 7A); moreover, phosphorylation was clearly attenuated upon introduction of LY294002. Furthermore, treatment of cells with neomycin, but not with calpeptin, strongly inhibited this phosphorylation.

Involvement of m-calpain in morphological and cytoskeletal remodeling in HUVECs in response to shear stress. How calpain modulates shear stress-induced HUVEC polarity was examined using interference reflection/GFP-actin microscopy (Fig. 5). Application of shear stress (30 dyne/cm²) to GFP-actin-expressing cells resulted in lamellipodial membrane protrusions, which lacked a preferential direction, within 10 min after shear onset (white arrowheads in Fig. 5A). The lamellipodia were more prominent in cells treated with PD98059 or U0126, suggesting that the ERK-pathway was required for activity.

Fig. 3. Mechanisms underlying shear stress-induced proteolytic activity in HUVECs. Cells loaded with bCAA-R110 were usually exposed to shear stress at 30 dyne/cm² with the exception of the shear stress-dependency experiment. Temporal F/F₀ changes in cells during the first 30 min following shear onset appear in the line graph. Maximal F/F₀ elevations (ΔF/F₀) during this period are represented in the bar graph. A: requirement of the ERK-pathway for activity. Cells were either pretreated with PD98059 (3–30 μM; PD98) or U0126 (0.3–3 μM; U01) for 15 min; subsequently, cells were sheared in the presence of the inhibitors at the matched concentrations. Results are expressed as the means ± SE (n = 3). *P < 0.05 compared with control groups. B: requirement of the PI3K-mediated pathway for activity. In the neomycin experiment, cells were pretreated with neomycin (0.1–10 mM) for 20 min, after which cells were sheared with normal KHB (Neo). In the LY294002 experiment, cells were pretreated with LY294002 (1–10 μM) for 30 min, after which cells were sheared in the presence of LY294002 at the matched concentrations (LY29). In the U73122 experiments, cells were pretreated with U73122 (1–10 μM) for 10 min and were sheared in the presence of U73122 at the matched concentrations (U73). Results are expressed as the means ± SE (n = 4). *P < 0.05 compared with control group.

Fig. 4. Effects of inhibitors on shear stress-induced phosphorylation of Akt in HUVECs. Phosphorylated Akt (P-Akt) or total Akt (Akt) expression level was determined by Western blot analysis. Cells were subjected to shear stress for 30 min (Shear). In terms of calpeptin administration, cells were pretreated with 1 μM calpeptin for 60 min, after which cells were sheared in the presence of calpeptin. LY294002 (LY29; 1 μM) and neomycin (Neo; 10 mM) were administered to the cells in a manner similar to that of Fig. 3. Shown blots are from the same nitrocellulose membrane. The bar graph illustrates the densitometric analysis of the blots. Phosphorylation of Akt, which was normalized by Akt expression, is expressed as the multiple increases relative to the control. Results are expressed as the means ± SE (n = 3). *P < 0.05 compared with static control group. #P < 0.05 compared with shear stress group.
podia in the downstream regions, which enlarged upon continuous application of shear, were enriched with well-developed stress fibers. Furthermore, IRM revealed decayed and newly constructed FAs, respectively, in the upstream and downstream regions of the cells; additionally, FAs were highly polarized toward the shear direction within 50 min following shear onset (Fig. 5, A and B). Calpeptin-treated cells also exhibited initial lamellipod protrusions within 10 min after shear onset (white arrowheads in Fig. 5A); however, the treated cells refrained from further lamellipod development and stress fiber formation. IRM demonstrated that treatment of sheared cells with calpeptin leads to well-developed, highly stabilized FAs (Fig. 5, A and B). Similar effects on cytoskeleton and FAs were observed in LY294002-treated cells (Fig. 5, A and B). Quantification of IRM images indicated that relative area of stable FAs increased in the presence of either calpeptin or LY294002 (Fig. 6A). Furthermore, FAs in calpeptin-treated cells accumulated due to shear stress; moreover, FAs displayed equal distribution in upstream and downstream regions regardless of shear stimulus. In contrast, FAs in control cells were clearly polarized toward the downstream regions by shear (Fig. 6B), suggesting that FA occupancy in the upstream regions was elevated preferentially by calpeptin treatment. In addition, shear stress-induced FA polarization was inhibited in cells treated with LY294002 (Fig. 6B).

We then determined whether FA molecules in sheared HUVECs are proteolytically cleaved by calpain (Fig. 7). Calpain-mediated cleavage of full-length vinculin (~120 kDa) and accumulation of a 95-kDa fragment in ionomycin-stimulated platelets were described previously (33). Similar accumulation was observed in ionomycin-stimulated HUVECs in the current study (Fig. 7A); moreover, accumulation was clearly attenuated following the introduction of calpeptin. Furthermore, application of shear stress up to 10 dyne/cm² for 30 min resulted in accumulation of the 95-kDa fragment (Fig. 7B), which was impaired by treatment with calpeptin (Fig. 7A). Shear stress also elicited calpeptin-sensitive reduction of full-length talin (220 kDa; Fig. 7C); on the other hand, FAK was unaltered by shear (Fig. 7D).

The effects of calpain silencing on shear stress-induced HUVEC alignment were assessed (Fig. 8). In static culture, HUVECs, which exhibited a cobblestone appearance, were randomly aligned (Fig. 8A). Prolonged application of shear stress (30 dyne/cm², 24 h) to control or m-calpain-knockdown cells led to an elongated spindle cell shape, as well as cell alignment in the direction of shear. This shear stress-induced alignment was markedly inhibited in m-calpain knockdown cells (Fig. 8B). The effects of m-calpain silencing on shear stress-induced FA-cytoskeletal remodeling were evaluated (Fig. 8C). In static culture, vinculin staining was evenly distributed in peripheral and central regions of control and m-calpain-knockdown cells in a dotlike pattern. Prolonged application of shear stress (30 dyne/cm², 24 h) to control cells led to polarized
vinculin distribution, which was characterized by localization of streaklike vinculin in the upstream and downstream regions. This vinculin polarity was clearly impaired in m-calpain-knockdown cells. Moreover, application of shear stress resulted in reorganization of actin cytoskeleton, which was characterized by well-developed, aligned F-actin stress fibers in central regions of the cells. Additionally, m-calpain silencing abrogated cytoskeletal reorganization.

**DISCUSSION**

Selective m-calpain activation in HUVECs in response to shear stress. The current data indicate that proteolytic cleavage of bCAA-R110 in HUVECs is promoted under physiological shear conditions (Fig. 2A) with no alteration of μ/H9262- or m-calpain expression levels (Fig. 1B). In addition, our preliminary data obtained using real-time RT-PCR demonstrated that at least m-calpain mRNA expression levels in HUVECs were unchanged by shear stimulus (data not shown). We thus attempted to demonstrate an isozyme-selective association between calpain and proteolytic activity; however, the most likely calpain inhibitors, which generally possess low isozyme specificity, reportedly inhibit serine protease or other cysteine proteases, as well as calpain (31). Consequently, the effects of siRNA-induced silencing of calpain isozymes on proteolytic activity in sheared HUVECs were assessed in this study. Selective silencing of m-calpain, but not of μ-calpain, suppressed shear stress-induced activity (Fig. 1D). This specific impairment is most likely not attributable to lack of μ-calpain-sensitivity of bCAA-R110, as ionomycin-induced proteolytic activity was partially and equally attenuated in both μ- and m-calpain-knockdown cells (See supplemental Fig. 3 in the online version of this article.). Furthermore, shear stress-induced calpain activity was further predicted by results obtained from Western blot analysis (Fig. 7). These findings, in concert, strongly suggest that m-calpain is mainly associated with shear stress-induced proteolytic activity in the cells.

Critical role of Ca²⁺ influx- and PI3K-mediated pathways for shear stress-induced calpain activity. A previous investigation suggested that the activity of μ-calpain, but not that of the m-isoform, accumulated through Ca²⁺ influx in interferon-γ-inducible protein 9-stimulated keratinocytes (32). Conversely, treatment of fibroblast cells with EGF caused selective m-calpain activation in a [Ca²⁺]i-independent manner (18). Thus, [Ca²⁺]i changes in living cells are generally believed to influence preferentially μ-calpain rather than m-calpain activity. However, perhaps surprisingly, the current data demonstrate that m-calpain-mediated proteolytic activity in sheared HUVECs is promoted through extracellular Ca²⁺ influx in-
volving nonselective cation channels (Fig. 2B), which suggests that the $[\mathrm{Ca}^{2+}]_i$ requirement for m-calpain activation is dependent on the types of stimulants or cells.

Earlier enzymatic investigations suggested that, at a minimum, submillimolar concentrations of $[\mathrm{Ca}^{2+}]_i$ were necessary for m-calpain activation (20). Several lines of evidence, however, indicated that $[\mathrm{Ca}^{2+}]_i$ in sheared ECs was far below submillimolar levels (22, 38). We hypothesized that these observations imply the presence of other mechanism(s) so as to lessen the $\mathrm{Ca}^{2+}$ requirement of m-calpain in sheared HUVECs. A previous report documented mediation of $[\mathrm{Ca}^{2+}]_i$ sensitization of m-calpain, which occurred in EGF-stimulated fibroblast cells, through ERK-induced direct phosphorylation of m-calpain (18); moreover, this event was required for subsequent calpain activation and cell motility (19). In addition to ERK, PIP$_2$, a membrane phospholipid, is thought to facilitate calpain activity in EGF-stimulated fibroblast cells via direct binding to m-calpain (35). Our data presented here showed that PIP$_2$ chelator suppressed shear stress-induced proteolytic activity in HUVECs; in contrast, activity was partially inhibited by ERK kinase inhibitor (Fig. 3), suggesting that the proteolytic activity is facilitated mainly through PIP$_2$-dependent pathway, although the ERK-mediated pathway plays a role. In addition, PLC (29) and PI3K (9), which can potentially influence inositol phospholipid metabolism, are reportedly activated in sheared ECs; consequently, we investigated whether these enzymes participate in the shear stress-induced proteolytic activity. Surprisingly, proteolytic activity was suppressed by PI3K inhibition but not by PLC inhibition (Fig. 3B). On the other hand, PI3K-dependent phosphorylation of Akt, which was induced by shear, was markedly inhibited by PIP$_2$ chelation but not by calpain inhibition (Fig. 4), indicating that m-calpain is activated downstream of PI3K; furthermore, neomycin-induced inhibition of proteolytic activity may be due to a reduction in the supply of PIP$_2$ to the PI3K pathway in large part. Therefore, these findings suggested that m-calpain activity in sheared HUVECs is facilitated mainly through the PI3K pathway.

A previous investigation revealed that PIP$_2$-mediated promotion of m-calpain activity in EGF-stimulated fibroblast cells is impaired upon introduction of exogenous PLC (34). Our current results similarly indicated that U73122-induced inhibi-
tion of endogenous PLC tends to promote shear stress-induced proteolytic activity in HUVECs (Fig. 3B). Presumably, these observations suggest that PI3K-mediated direct sensitization can modify calpain activity even in HUVECs; however, additional studies are necessary to identify the contribution of this mechanism with respect to the shear stress-induced signaling pathway.

Association of m-calpain activity with shear stress-induced regulation of HUVEC shape. An earlier investigation suggested that disassembly of FA molecules paxillin, vinculin, and zyxin in fibroblast cells is dependent on the ability of calpain to cleave talin (12). Consequently, talin cleavage appears to be a rate-limiting step of calpain-mediated FA turnover. In contrast to talin, the role of vinculin in calpain-mediated FA turnover remains poorly understood. However, vinculin is generally believed to stabilize FAs based on enhanced migration in vinculin-null cells (5). FAK is also a calpain substrate (6); moreover, FAK is thought to be associated with mechanosignaling, as well as with the motility process in ECs (26). Indeed, FAK-null cells exhibit impaired cell polarity and movement (23). In this study, our results directly demonstrated, for the first time, the proteolytic cleavage of vinculin and talin, but not of FAK, in response to shear stress, which was clearly attenuated by calpain inhibition (Fig. 7). Collectively, it appears that shear stress-induced calpain activity, in all likelihood, tends to destabilize FAs.

In subconfluent ECs, newly formed FAs appear in the leading edges within a few minutes following shear onset (25). After development of the initial lamellipod protrusions, FAs located at the tail edges of the cells are believed to undergo destabilization and release. This rear detachment reportedly facilitates further lamellipod development and forward movement of the cell body (25). As a result of lamellipod development and detachment, FAs in the cells are thought to polarize toward flow directions. The visualized data of the current investigation revealed that shear stress elicited similar FA and cytoskeletal reorganization of HUVECs (Fig. 5). The pharmacological inhibition of calpain or PI3K prevented shear stress-induced destabilization and polarization of FAs without altering initial lamellipod protrusions (Fig. 6); thus, the PI3K/m-calpain pathway appears to be necessary for modulation of shear stress-induced FA turnover by potentially destabilizing preexisting FAs and/or limiting de novo FA formation, particularly in the upstream regions. This finding is consistent with that of our previous report, which demonstrated the association between fluid flow-induced localized [Ca²⁺], transients in ECs and rear retractions and termination of lamellipod extensions (28).

Prolonged application of physiological shear stress to confluent ECs reportedly causes reorganization of actin cytoskeleton and cell alignment in the direction of the shear stress with polarized vinculin and α5β3 integrin distribution (17); thus, this remodeling is considered to play a pivotal role in terms of shear stress-induced changes in cell shape. The essential contribution of PI3K and calpain, as well as Rho kinase to remodeling, was pharmacologically identified in the earlier investigation (4); however, the exact mechanisms underlying PI3K- or calpain-mediated signaling remain poorly understood. The present study established that m-calpain activity, which is downstream of PI3K, is required for shear stress-induced initial FA detachment (Figs. 5 and 6) and subsequent EC alignment (Fig. 8). On the other hand, the dominant role of Rho kinase in shear stress-induced EC remodeling was documented previously (4, 26). Rho kinase, in general, is activated by shear stimulus, resulting in the generation of actomyosin-based contractile force, particularly in the rear of shear stress-induced migrating ECs, which physically facilitates FA detachment (26). Thus, m-calpain may biochemically modulate shear stress-induced EC alignment by eliciting FA detachment, presumably coordinating Rho-induced contractile force at the rear of the cell.

In conclusion, the current findings afforded novel insights regarding m-calpain activity in sheared HUVECs. These data revealed that m-calpain activity is triggered by Ca²⁺ influx and promoted mainly through the PI3K pathway. This activity is necessary for modulation of FA polarization and subsequent cell alignment, likely through selective cleavage of FA molecules during the early phase of mechanotransduction.

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REFERENCES


