An Ezrin/Calpain/PI3K/AMPK/eNOS$_{s1179}$ Signaling Cascade Mediating VEGF-Dependent Endothelial Nitric Oxide Production

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***Abstract*—**Calpain was recently reported to mediate vascular endothelial growth factor (VEGF)-induced angiogenesis. In the present study, we investigated detailed molecular mechanisms. VEGF (100 ng/mL) induced a marked increase in endothelial cell production of NO$^{\bullet}$, specifically detected by electron spin resonance. This response was abolished by inhibition of calpain with *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) or Calpeptin. Both also diminished membrane-specific calpain activation by VEGF, which was intriguingly attenuated by silencing ezrin with RNA interference. A rapid membrane colocalization of calpain and ezrin occurred as short as 10 minutes after VEGF stimulation. AKT, AMP-dependent kinase (AMPK), and endothelial nitric oxide synthase (eNOS)$_{s1179}$ phosphorylations in VEGF-stimulated endothelial cells were markedly enhanced, which were however significantly attenuated by either ALLN, Calpeptin, or ezrin small interfering RNA, as well as by Wortmannin or compound C (respectively for phosphatidylinositol 3-kinase [PI3K] or AMPK). The latter 3 also abolished VEGF induction of NO$^{\bullet}$. These data indicate that AMPK and AKT are both downstream of PI3K and that AKT activation is partially dependent on AMPK. The interrelationship between AMPK and AKT, although known to be individually important in mediating VEGF activation of eNOS, is clearly characterized. Furthermore, AMPK/AKT/eNOS$_{s1179}$ was found downstream of a calpain/ezrin membrane interaction. These data no doubt provide new insights into the long mystified signaling gap between VEGF receptors and PI3K/AKT or AMPK-dependent eNOS activation. In view of the well-established significance of VEGF-dependent angiogenesis, these findings might have broad and important implications in cardiovascular pathophysiology. **(*Circ Res*. 2009;104:50-59.)**

Key Words: calpain ■ ezrin ■ nitric oxide ■ vascular endothelial growth factor ■ eNOS

Calpain is a cytosolic cysteine protease that translocates to membrane on activation. Active calpain not only cleaves its substrates but also disrupts protein interactions. Various kinases, phosphatases, and cytoskeletal proteins are known substrates for calpain.[1–3] Through interactions with its substrates, calpain plays an important role transducing signals of cell migration, differentiation, and proliferation.[3–6] Specifically in endothelial cells however, limited knowledge exists regarding how calpain signals to modulate cell functions. Interestingly, a critical role of calpain in mediating vascular endothelial growth factor (VEGF)-induced angiogenesis was recently reported, despite unknown molecular mechanisms.[7] Because VEGF-mediated angiogenesis is at least partially attributed to endothelial cell production of nitric oxide (NO$^{\bullet}$),[8,9] we became interested in whether some of the undiscovered roles of calpain deal with VEGF production of NO$^{\bullet}$ and angiogenesis. Using the highly specific and sensitive electron spin resonance (ESR) to measure nitric oxide radical (NO$^{\bullet}$), we examined for the first time whether calpain is required for VEGF stimulation of endothelial NO$^{\bullet}$ production. Changes in membrane and cytosol-specific activities of calpain, and their relevance to downstream signaling, were studied in depth.

Ezrin is a member of the ezrin/radixin/moesin (ERM) protein complex that is classically involved in cytoskeletal remodeling. Ezrin and other ERM proteins act as linkers between plasma membrane and cytoskeletal proteins.[10] On conformational changes, ezrin can actively participate in transducing cytoskeletal signals[11,12] to modulate a wide variety of cellular functions besides serving as structural linkers.[13,14] However, potential roles of ezrin signaling in modulating endothelial cell function have remained largely unknown. We hypothesized that ezrin exerts its regulatory roles via targeted interaction with calpain. In the present study, ezrin-dependent calpain localization to membrane, and a potential role of ezrin in calpain-mediated endothe-

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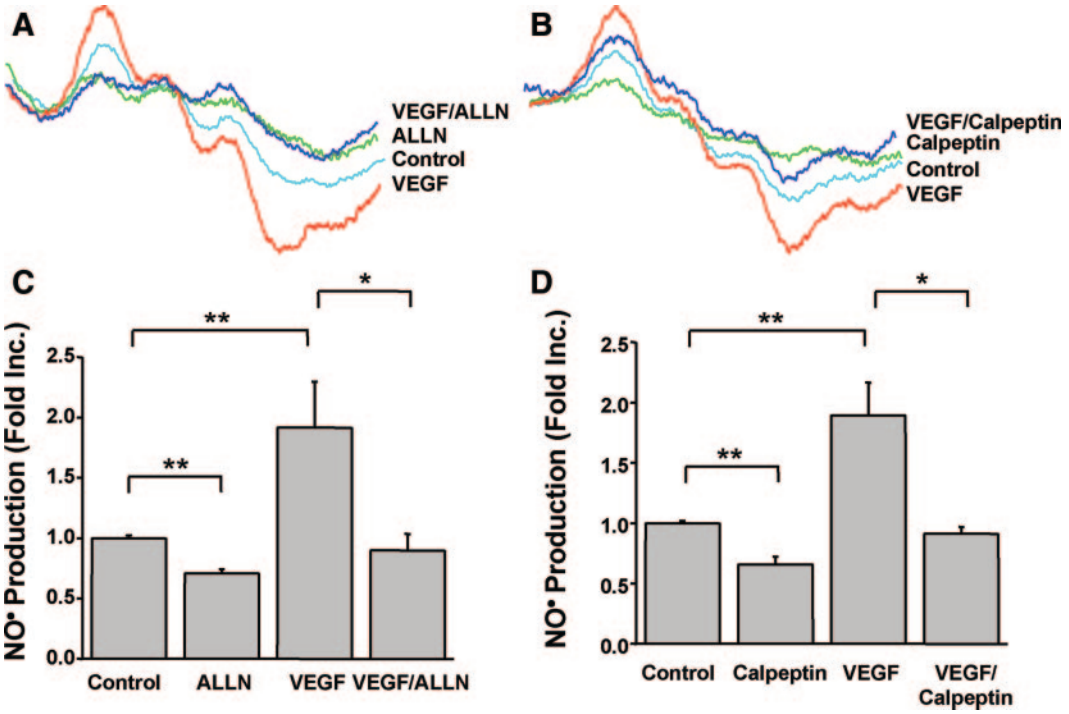

Figure 1. Calpain plays a critical role in basal and VEGF-stimulated endothelial cell production of NO•. Endothelial cells were preincubated with Calpeptin (10 μmol/L, 30 minutes) or ALLN (30 μmol/L, 30 minutes) before VEGF stimulation (100 ng/mL) and analysis of NO• production using an ESR spectrophotometer. A and B, Representative ESR spectra. C and D, Grouped densitometric data. Means±SEM; ANOVA; *$P<0.05$, **$P<0.01$.

lial NO• production in response to VEGF, were thoroughly investigated using immunocytochemical, coimmunoprecipitation and RNA interference approaches.

VEGF is one of the most potent angiogenic factors, and its signaling is crucial for both angiogenesis and vasculogenesis.[8,15–17] VEGF potently induces NO• production through AKT and/or AMPK-dependent phosphorylation of endothelial nitric oxide synthase (eNOS).[17–26] VEGF-stimulated NO• not only directly enhances endothelial cell migration and proliferation but also modulates effects of numerous other angiogenic factors that might work in concert with VEGF to promote angiogenesis.[16] Indeed, selective inhibition of eNOS prevents VEGF-mediated endothelial migration, proliferation, and capillary-like network formation. On the other hand, a decrease in NO• bioavailability is related to vascular dysfunction.[8,9,21,27,28] From these reports, it has been suggested that selective modulation of eNOS activity can be provided as an attractive strategy for regulation of angiogenesis and vascular permeability. Nonetheless, precise signaling mechanisms underlying VEGF activation of eNOS and endothelial NO• production remain incompletely understood. Besides phosphatidylinositol 3-kinase (PI3K)/AKT and/or AMPK, it has remained unclear whether and how alternative or parallel signaling cascades participate in VEGF activation of eNOS.

Therefore, in the present study, we fully characterized novel roles of ezrin and calpain in VEGF activation of eNOS and the potential interrelated roles of AKT and AMPK in transducing ezrin/calpain signals to eNOS. We found that ezrin-dependent, membrane-specific translocation and activation of calpain by VEGF precedes AMPK and AKT-dependent phosphorylation of $eNOS_{s1179}$ and production of NO•. AKT is activated downstream of PI3K similarly to AMPK, but is also partially dependent on AMPK. These observations not only provide new information as to how AMPK and AKT interacts to ensure VEGF induction of NO• but also represent the first evidence establishing the critical role of ezrin/calpain interaction in modulating endothelial cell function. Because the newly characterized signaling cascade of ezrin/calpain/PI3K/AMPK/eNOS likely has fundamental roles in cell signaling in various other cell types, these novel observations may have broad applicability to both vascular and cancer pathophysiology.

Methods and Materials

Materials

Phospho-AMPK (Thr172), pan-α-AMPK, and phospho-AKT (Ser473), phospho-eNOS (Ser1179), phospho-p44/p42 (Thr202/Tyr204), PI3K p110, PI3K p85, phospho-PKC (pan; Ser660), phospho-PKC (Thr505), heat shock protein (HSP)90, ezrin, and phospho-eNOS (Thr495) antibodies were purchased from Cell Signaling Technologies (Beverly, Mass). Phospho-eNOS (Ser116) antibody was obtained from Upstate (Charlottesville, Va). Anti–M-calpain antibody was purchased from Affinity BioReagent (ABR Inc, Golden, Colo). β-Actin antibody and secondary antibodies were purchased from Sigma and Bio-Rad, respectively. VEGF was purchased from R&D Systems. Wortmannin, compound C, Calpeptin and *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) were obtained from Calbiochem (La Jolla, Calif). Oligofectamine and Opti-MEM for small interfering (si)RNA transfection were purchased from Invitrogen (Carlsbad, Calif). Other chemicals were obtained from Sigma in highest purity.

Cell Culture

Bovine aortic endothelial cells (Cell Systems, Kirkland, Wash) of passages 4 to 6 were cultured in medium 199 (Invitrogen), containing 10% FBS to confluence and starved in 5% media overnight before experiments.

Membrane Fractionation

Cells were harvested with iced-cold PBS and centrifuged for 5 minutes at 1000 rpm. Cell pellets were resuspended in homogeniza-

tion buffer (Tris 50 mmol/L, EDTA 0.1 mmol/L, EGTA 0.1 mmol/L, protease inhibitor cocktail, pH 7.4) and sonicated on ice (5 seconds, 10×) to lyze the cells. After centrifugation at 3500 rpm for 5 minutes, the supernatant was transferred to a fresh tube and centrifuged for 90 minutes at 50 000 rpm. The pellets (membrane fraction) were resuspended in lysis buffer and the cytosolic fraction (supernatant) transferred into a fresh tube.

Calpain Activity Assay

Calpain protease activity was determined using a calpain activity kit (Calbiochem QIA120). Fifty microliters each of serially diluted standards, controls, or samples were mixed with 5 μL of synthetic substrate for calpain, Suc-LLVY-AMC, and 100 μL activation buffer or inhibition buffer, before incubation for 15 minutes at 37°C. The calpain-dependent cleavage product of 7-amido-4-methyl coumarin (AMC) was measured fluorometrically at excitation and emission wavelengths of 360 to 380 nm and 440 to 460 nm, respectively. The calculated calpain activity was determined by subtracting the activity readings obtained using the inhibition buffer, from the activity readings detected with the activation buffer. The specific activity is presented as product formation at micromole per liter per mg protein per minute after calculation by the AMC standard curve.

ESR Detection of Endothelial NO$^{\bullet}$ Production

Bioavailable NO$^{\bullet}$ produced by confluent endothelial cells was detected using ESR, as we described previously.[27,28]

siRNA Transfection

Proliferating endothelial cells of 85% to 90% confluence were transfected with control siRNA or ezrin siRNA (25 nmol/L, Dharmacon Inc; ezrin sequence: 5′-ACAGCGCCAUGCUGGAAUAUU-3′) using Oligofectamine for 4 hours in Opti-MEM serum-reduced media before addition of growth media containing 10% FBS. Forty-eight hours later, cells were subjected designated assays.

Western Blot Analysis of Protein Phosphorylations

Cells were lysed in iced-cold Tris buffer (50 mmol/L Tris, pH 7.4, 2 mmol/L EDTA, 1 mmol/L EGTA) containing 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 50 mmol/L NaF, 10 mmol/L $Na_4P_2O_7$, 1 mmol/L Na_3VO_4, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μL/mL the protease inhibitor cocktail, and 10 μL/mL the phosphatase inhibitor cocktail stock solution (Sigma, St Louis, Mo). Lysate proteins were separated in 7.5% SDS-PAGE. Blots were subjected to incubations with primary antibodies (mostly at 1:1000, β-actin at 1:5000) overnight at 4°C and peroxidase-conjugated secondary antibodies at room temperature for 1 hour, and phosphorylated proteins were subsequently visualized using ECL. Band intensities were analyzed using Image J program and normalized against β-actin.

Immunocytochemical Staining

Cells grown on cover slide were treated with VEGF for 10 minutes, washed with PBS twice, and fixed in cold 4% paraformaldehyde for 20 minutes and then permeabilized with 0.1% Triton X-100 in PBS for 20 minutes. After quenching for 10 minutes with 50 mmol/L NH_4Cl, cells were blocked with PBS containing 5% goat serum. Primary antibodies (calpain, 1:250; ezrin, 1:250) were added for 1-hour incubation sequentially at room temperature. After washing, cells were incubated with secondary antibodies at room temperature for 1 hour (Alexa Fluor 488 for calpain, Alexa Fluor 594 for ezrin in sequence). After washing with PBS for 3 times, cells were mounted on slides using ProLong Antifade (Invitrogen). The fluorescent images were captured using a Leica TCS-SP Confocal Microscope and analyzed with the LCS Lite software.

Immunoprecipitation

For immunoprecipitation, cells were lysed in mild lysis buffer (50 mmol/L Tris-Cl pH 8.0, 150 mmol/L NaCl, 1% NP-40, 0.1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, and 5 μg/mL leupeptin) and preincubated with protein A/G-agarose beads for 2 hours at 4°C. Samples were then centrifuged, and supernatants subjected to immunoprecipitation with 2 μg of antibody overnight. The protein A/G-agarose beads (Calbiochem) were added for a further 16-hour incubation at 4°C, and immunoprecipitates were used for Western blotting.

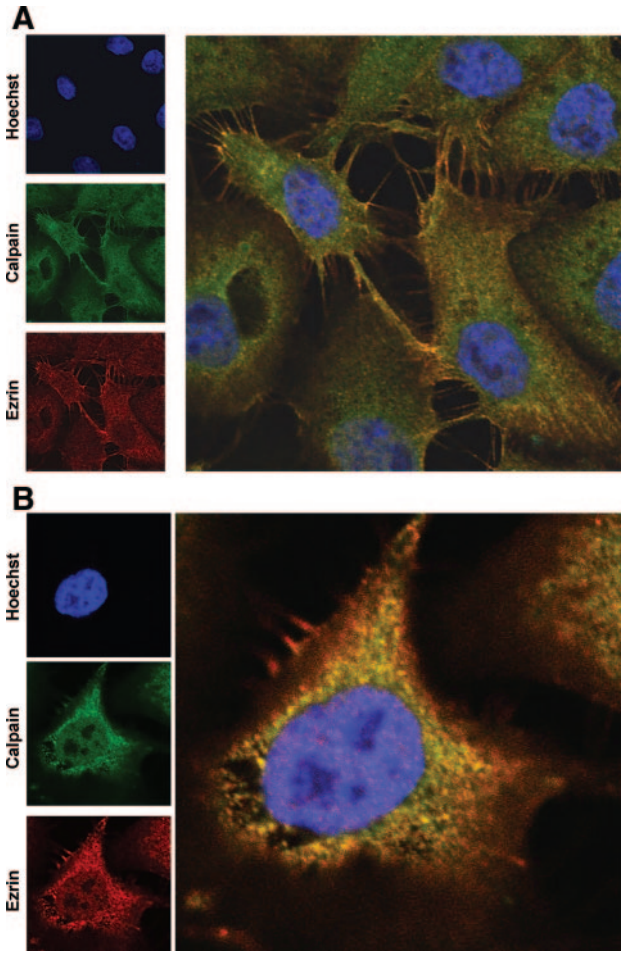

Figure 2. Colocalization of calpain and ezrin in response to VEGF. Endothelial cells cultured on coverslips were treated with VEGF for 10 minutes before immunostaining with fluorescent antibodies. Calpain and ezrin were visualized in green and red, respectively, whereas nDNA (in blue) was stained with Hoechst 33258. A and B, Representative subcellular localization of calpain and ezrin in unstimulated and VEGF-stimulated endothelial cells.

Statistical Analysis

All data are presented as means±SEM from 4 to 8 independent experiments. ANOVA was used to compare means of different experimental groups, and Dunnet and Tukey tests were used as post tests. A probability value less than 0.05 was considered significant.

Results

A Critical Role of Calpain in Basal and VEGF-Stimulated NO$^{\bullet}$ Production From Endothelial Cells

It has been previously established that VEGF activates eNOS to result in an increase in endothelial NO$^{\bullet}$ production. These previous studies either examined mechanisms underlying enzymatic activations of eNOS, or an intermediate role of NO$^{\bullet}$, in VEGF-provoked therapeutic angiogenesis. Production of NO$^{\bullet}$

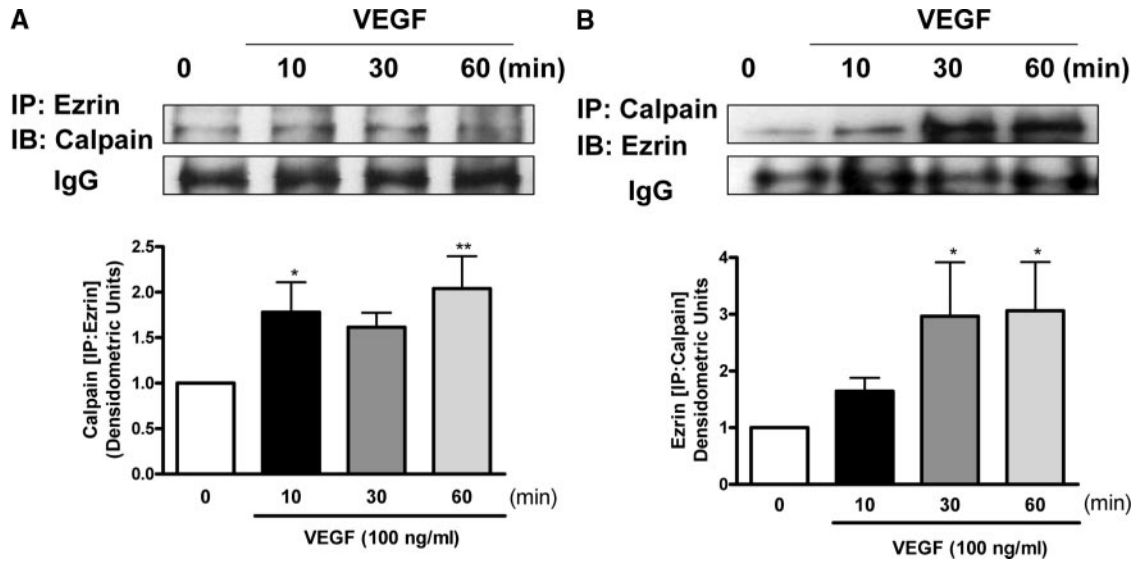

Figure 3. Coimmunoprecipitation of calpain and ezrin. Endothelial cells were stimulated with 100 ng/mL VEGF for indicated times. A, Cell lysates were subjected to immunoprecipitation with ezrin antibody, followed by immunoblotting with calpain. B, Cell lysates were subjected to immunoprecipitation with calpain antibody, followed by immunoblotting with ezrin. Grouped quantitative data are presented as means±SEM (n=4); ANOVA; *$P<0.05$, **$P<0.01$.

was assessed by either its metabolites nitrate and nitrite or in vitro enzymatic analysis of eNOS. In the present study, we measured bioavailable $NO^{\bullet}$ specifically using ESR, as previously described.[29,30] Indeed, exposure of cells to VEGF (100 ng/mL, 1 hour of incubation with $NO^{\bullet}$-specific spin trap $Fe^{2+}(DETC)_2$ colloid following VEGF treatment) induced a marked and reproducible increase in $NO^{\bullet}$ production (Figure 1).

Intriguingly, in endothelial cells preincubated with inhibitors for the cysteine protease calpain, ALLN (30 μmol/L, 30 minutes) or Calpeptin (10 μmol/L, 30 minutes), basal and VEGF-stimulated $NO^{\bullet}$ productions were significantly attenuated, as demonstrated by representative ESR spectra and grouped densitometric data (Figure 1). This calpain-dependent $NO^{\bullet}$ production, as well as calpain-dependent phosphorylations of $eNOS_{s1179}$, AKT and AMPK, were also reproducible in response to a lower, physiological concentration of VEGF (10 ng/mL; supplemental Figure I, A through C, and supplemental Figure II, A through D). These data indicate a novel role of calpain in maintaining basal eNOS activity and mediating VEGF activation of eNOS. Detailed signaling events downstream of VEGF activation of calpain, connecting to eNOS activation, have been studied in depth (see below).

Membrane-Specific Activation of Calpain by VEGF and Its Dependence on Colocalization With Ezrin

Under basal conditions, calpain is predominantly situated in cytosol. In VEGF-stimulated endothelial cells, total cellular calpain activity was significantly increased by VEGF treatment (100 ng/mL, 10 minutes) but abolished by preincubation with Calpeptin or ALLN (10 or 30 μmol/L, 30 minutes) (supplemental Figure III A). Whereas membrane-specific calpain activity was similarly enhanced by VEGF and attenuated by the inhibitors, cytosolic activity of calpain remained unchanged (supplemental Figure III, B and C). These data seem to suggest that membrane-specific activation of calpain is important for acute VEGF signaling. Interestingly, immunocytochemical staining of VEGF-stimulated cells with calpain, ezrin, and secondary fluorescent antibodies revealed enhanced membrane colocalization (Figure 2A and 2B). This augmented colocalization was further confirmed by coimmunoprecipitation experiments pulling down calpain or ezrin alternatively (Figure 3A and 3B). VEGF induced a time-dependent association between calpain and ezrin, which occurred as early as 10 minutes, whereas lysates pulled down with control IgG showed no difference (Figure 3A and 3B).

Role of Ezrin in VEGF Stimulation of Endothelial $NO^{\bullet}$ Production

Data described above indicate a critical role of ezrin in VEGF activation of calpain. To investigate a specific role of ezrin in calpain-dependent endothelial $NO^{\bullet}$ production, cells were transfected with ezrin siRNA with or without Calpeptin. In control siRNA–transfected cells, VEGF-stimulated $NO^{\bullet}$ production similarly as in untransfected cells, which was abolished by Calpeptin (Figure 4A). Transfection with ezrin siRNA attenuated $NO^{\bullet}$ production, although Calpeptin was able to further reduce it (Figure 4A). Ezrin protein expression, $eNOS_{s1179}$, and AMPK phosphorylations were all attenuated by ezrin siRNA transfection, as shown by representative blots and quantitative data (Figure 4B through 4D), whereas the siRNAs had no effects on calpain, eNOS, AKT, or AMPK protein levels. Moreover, membrane-specific calpain activity was significantly decreased in VEGF-stimulated, ezrin siRNA–transfected cells (supplemental Figure IV), confirming a specific effect of ezrin on VEGF activation of calpain. Take together, these data demonstrate an essential role of ezrin in mediating VEGF activation of calpain and the consequent endothelial $NO^{\bullet}$ production.

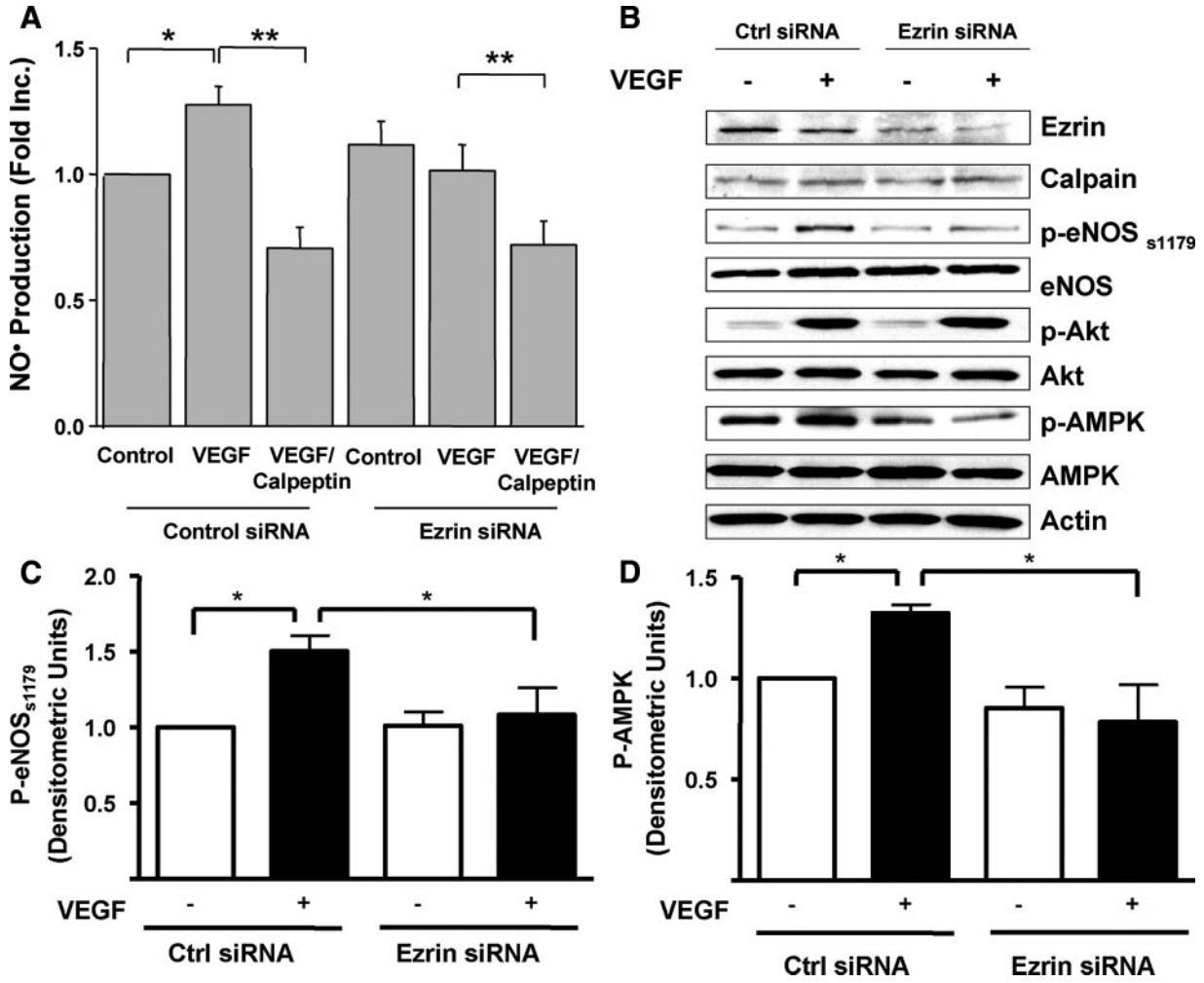

Figure 4. The role of ezrin in calpain-dependent endothelial NO• production in response to VEGF. Proliferating endothelial cells were transfected with 25 nmol/L control or ezrin siRNA before Calpeptin (10 μmol/L, 30 minutes) and VEGF (100 ng/mL, 60 minutes). A, Grouped data of NO• production are presented as Means±SEM. B, Representative Western blot from cells transfected with ezrin siRNA. C and D, Ezrin mediated phosphorylation of $eNOS_{s1179}$ and AKT in response to VEGF (10 minutes). ANOVA; *$P<0.05$, **$P<0.01$.

Calpain Is Required for PI3K/AKT-Dependent eNOS Phosphorylation in Response to VEGF

PI3K/AKT is known to mediate VEGF activation of eNOS. Indeed, VEGF increased AKT phosphorylation up to 4.3-fold, which was markedly attenuated by Calpeptin or ALLN (Figure 5A). Neither VEGF stimulation nor calpain inhibitors affected protein expression of PI3K subunits, indicating absence of genomic effects or calpain-dependent cleavage (data not shown). $eNOS_{s1179}$ phosphorylation in VEGF-stimulated or Calpeptin- or ALLN-preincubated endothelial cells mirrored changes in AKT phosphorylation (Figure 5B). Of note, VEGF stimulation also resulted in an increase in $eNOS_{s116}$ phosphorylation and a decrease in $eNOS_{t497}$ phosphorylation, both of which have been previously studied to be functional in modulating eNOS activity. However, Calpeptin or ALLN affected neither (Figure 5C and 5D). In additional experiments, calpain siRNAs were used to transfect endothelial cells before VEGF stimulation. Of note, calpain 2 (M-calpain) siRNA was effective in attenuating VEGF production of NO• (Figure 6A and 6B), as well as calpain expression, AKT, AMPK, and $eNOS_{s1179}$ phosphorylations (Figure 6C), further confirming a specific role of M-calpain in VEGF activation of eNOS.

Ezrin/Calpain Interaction Is Upstream of Parallel AMPK and AKT-Dependent VEGF Activation of eNOS

Intriguingly, in cells pretreated with compound C (20 μmol/L, 30 minutes), an inhibitor for AMPK, VEGF-stimulated endothelial NO• production was completely attenuated despite a similarly potent effect of the PI3K antagonist Wortmannin (100 nmol/L, 30 minutes) (Figure 7A and 7B). In subsequent experiments, VEGF-induced AMPK phosphorylation was abolished by Wortmannin (Figure 7C). AKT phosphorylation was partially attenuated by compound C (Figure 8B). These data suggest that AMPK has a previously uncharacterized role serving as an intermediate between PI3K and AKT in mediating eNOS activation. Furthermore, markedly increased AMPK phosphorylation in response to VEGF was dramatically attenuated by ALLN or Calpeptin, to a level lower than the baseline (Figure 7C). On the other hand, calpain activity was unaffected by compound C or H89, a protein kinase A inhibitor, excluding an

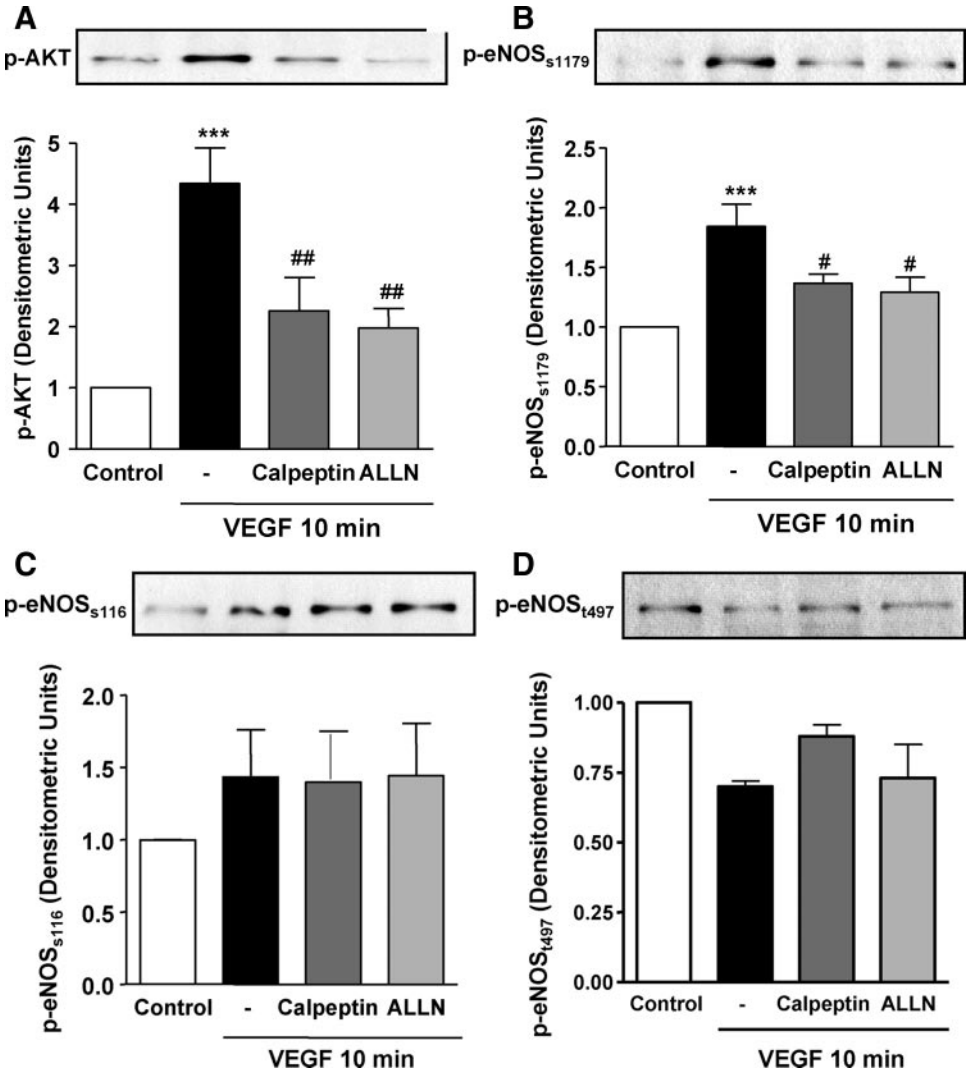

Figure 5. Calpain-dependent phosphorylation of AKT and eNOS in response to VEGF. Endothelial cells were pretreated with Calpeptin (10 μmol/L) or ALLN (30 μmol/L) for 30 minutes before VEGF stimulation (100 ng/mL, 10 minutes). A, AKT phosphorylation. B, eNOS$_{s1179}$ phosphorylation. C, eNOS$_{s116}$ phosphorylation. D, eNOS$_{t497}$ phosphorylation. Representative Western blots are shown with quantitative grouped data (means±SEM; n=5); ANOVA; ***$P<0.001$ vs control; #$P<0.05$, ##$P<0.01$ vs VEGF.

upstream role of AMPK or protein kinase A in calpain activation (Figure 7D). Thus, these data establish an novel role of calpain in VEGF activation of AMPK in endothelial cells. In the same groups of experiments, AMPK phosphorylation was abolished by compound C, confirming its specificity in endothelial cells (Figure 8A). eNOS$_{s1179}$ phosphorylation was consistently inhibited by Calpeptin (Figures 8C and 6B) and by Wortmannin or compound C (Figure 8C), demonstrating that calpain, PI3K, and AMPK all lie upstream of eNOS activation.

Of note, AKT phosphorylation was significantly inhibited by Wortmannin (Figure 8B), Calpeptin (Figure 8B and 8A), or compound C (Figure 8B). These data confirm the classic pathway of PI3K/AKT/eNOS that is activated on VEGF stimulation. Its dependence on calpain activation, however, is a new observation. Furthermore, partial dependence on AMPK of AKT phosphorylation seems to suggest a new intermediate role of AMPK connecting PI3K and AKT, besides its direct effects on eNOS activation.

Calpain Mediates Hydrogen Peroxide–Provoked Endothelial NO$^{\bullet}$ Production: Potential Link to Redox Sensitivity of VEGF Activation of Calpain

Reactive oxygen species (ROS), particularly superoxide and hydrogen peroxide (H_2O_2), have been shown to modulate endothelial cell function via interactions with NO pathways.[31–34] It has also been demonstrated to mediate some receptor signaling such as VEGFR activation and epidermal growth factor receptor transactivation in endothelial cells.[32,34–36] VEGF-induced membrane translocation of Rac1 from cytosol activates NADPH oxidases, which require Rac1 as a key regulator for activation.[34,35,37,38] NADPH oxidase–derived ROS can, in turn, participate in VEGF-stimulated angiogenesis.[34,38,39] Thus, we investigated whether endothelial NO$^{\bullet}$ production, provoked by H_2O_2, is calpain-dependent. As shown in supplemental Figure V, H_2O_2 increased NO$^{\bullet}$ production similar to what we found previously[40] and that calpain inhibitors, Calpeptin and ALLN, were effective in completely reversing this response. In contrast, calpain activity was not affected by bradykinin (100 nmol/L for 10 minutes; supplemental Figure VI, A). The specificity of the compounds in endothelial cells was confirmed (supplemental Figure VI, B). Taken together, these results seem to implicate that calpain-dependent activation of endothelial NO$^{\bullet}$ production is agonist-specific. Whether or not it is a ROS-dependent pathway, however, requires further investigations that are beyond the scope of the present study.

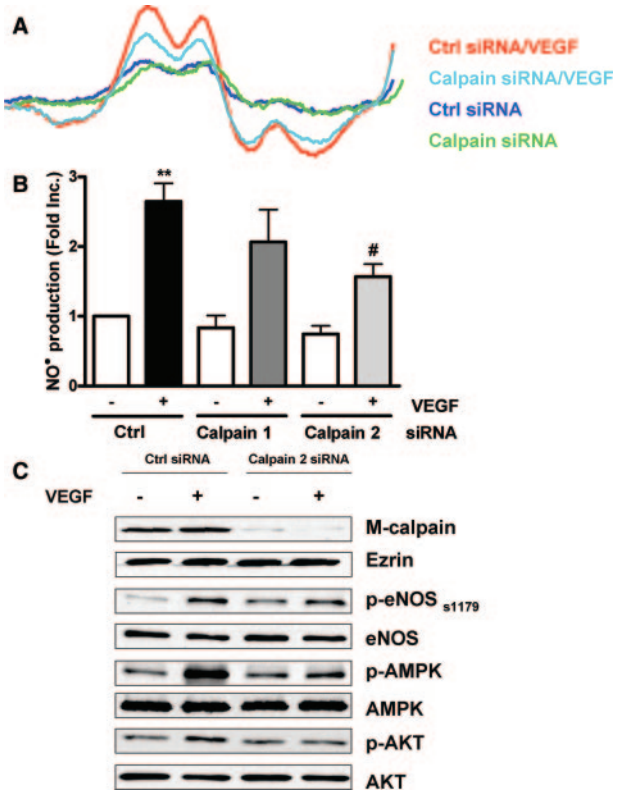

Figure 6. The specific role of calpain-2 on endothelial NO• bioavailability. BAECs were transfected with 25 nmol/L control siRNA or 25 nmol/L calpain 2 siRNA and then exposed to 100 ng/mL VEGF. A, Representative ESR spectra. B, Grouped densitometric data of NO• production. C, Representative Western blots. ANOVA; *$P<0.05$, **$P<0.01$.

The novel findings from the present study are summarized in a schematic format in supplemental Figure VII. On VEGF stimulation of endothelial cells, membrane-specific activation of calpain occurs ezrin-dependently, which, in turn, results in PI3K-dependent AMPK activation, consequent AKT and eNOS phosphorylations, and increased NO• production. AKT activation seems partially dependent on AMPK, establishing a crosstalk between PI3K/AMPK/eNOS and PI3K/AKT/eNOS pathways. Taken together, these data unravel an ezrin/calpain/PI3K/AMPK/AKT/eNOS$_{s1179}$ pathway that is critical for VEGF induction of endothelial NO• production, which would, in turn, mediate VEGF-dependent angiogenesis.

Discussion

The most significant finding of the present study is novel characterization of calpain/ezrin interaction in mediating AMPK-dependent eNOS activation in response to VEGF. These data represent the first evidence that calpain signaling, via interaction with ezrin, plays a critical role in modulating endothelial cell function, in particular, resulting in agonist-dependent NO• production. Besides the direct activating effect of AMPK on eNOS, our data also revealed a previously uncharacterized intermediate role of AMPK in linking PI3K activation to AKT-dependent eNOS$_{s1179}$ phosphorylation.

In the presence of calcium, purified calpain enzymes were found effective in cleaving eNOS that is pulled down from endothelial cell lysates using a monoclonal antibody for HSP90.[41] However, similar procedures indicated that such an incubation did not affect eNOS activity.[41] Regardless, these findings may suggest a possible mechanism by

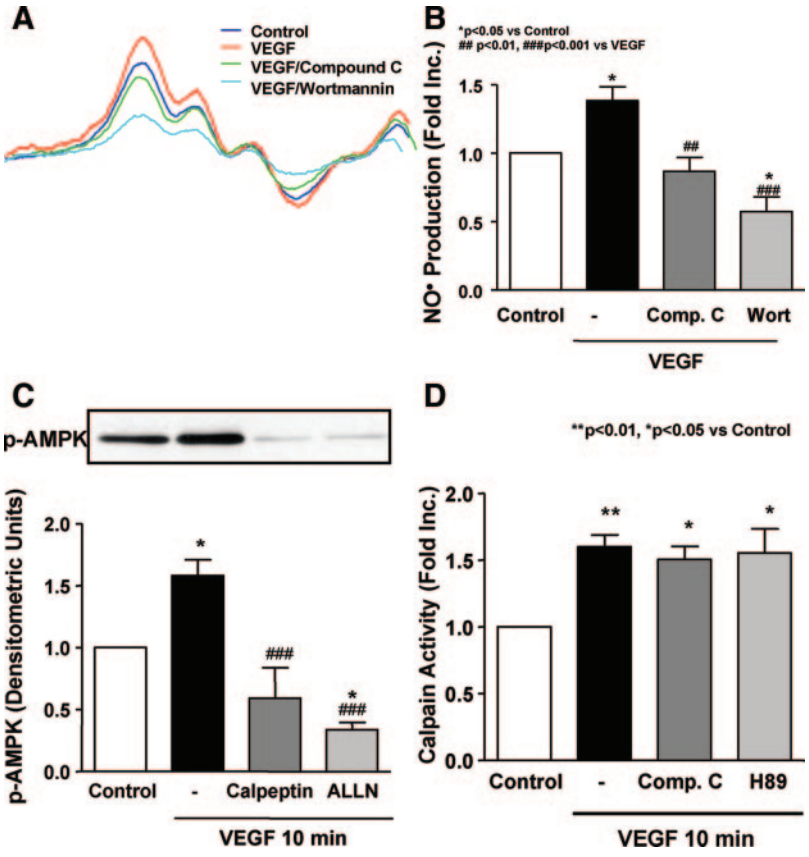

Figure 7. PI3K and AMPK are required for VEGF induction of NO•. Endothelial cells were preincubated with an AMPK inhibitor compound C (20 μmol/L, 30 minutes) or the PI3K antagonist Wortmannin (100 nmol/L, 30 minutes) before VEGF stimulation (100 ng/mL, 60 minutes) and analysis of NO• production. A, Representative ESR spectra. B, Grouped densitometric ESR data (means±SEM; n=4). C, AMPK phosphorylation at Thr172 from BAECs pretreated with calpain inhibitors, Calpeptin (10 μmol/L), or ALLN (30 μmol/L) for 30 minutes before VEGF (100 ng/mL, 10 minutes). D, Calpain activity from cells preincubated with a selective AMPK inhibitor, compound C (20 μmol/L, 30 minutes), or H89 (10 μmol/L, 30 minutes) before 100 ng/mL VEGF stimulation for 10 minutes. Grouped data are presented as means±SEM (n=5). ANOVA; *$P<0.05$, **$P<0.01$ vs control.

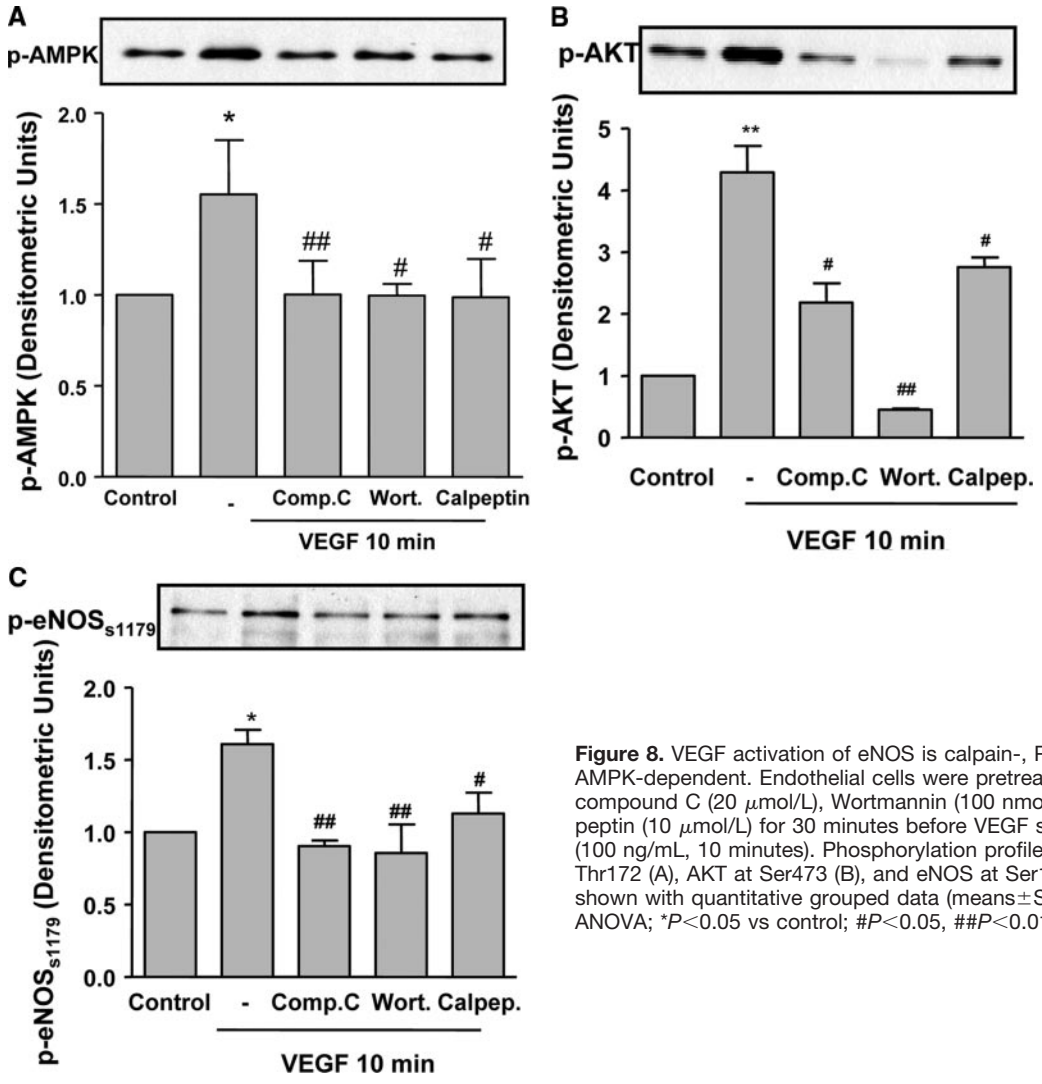

Figure 8. VEGF activation of eNOS is calpain-, PI3K-, and AMPK-dependent. Endothelial cells were pretreated with compound C (20 μmol/L), Wortmannin (100 nmol/L), or Calpeptin (10 μmol/L) for 30 minutes before VEGF stimulation (100 ng/mL, 10 minutes). Phosphorylation profiles of AMPK at Thr172 (A), AKT at Ser473 (B), and eNOS at Ser1179 (C) are shown with quantitative grouped data (means±SEM, n=4). ANOVA; *$P<0.05$ vs control; #$P<0.05$, ##$P<0.01$ vs VEGF.

which calpain indirectly affects eNOS function, such as via altering eNOS interactions with chaperon proteins. In our VEGF-stimulated endothelial cells, however, HSP90 association with eNOS was unaffected (by coimmunoprecipitation experiments; data not shown), seemingly suggesting that such a mechanism is unlikely involved in the calpain-dependent eNOS activation in response to VEGF. By contrast, our data demonstrated a novel role for cytoskeletal protein ezrin in modulating membrane localization and activation of calpain; thus, ezrin is involved in calpain-dependent eNOS activation. Of interest, in NIH 3T3 cells, inhibition of calpain was found to be associated with increased ezrin levels and enhanced cell spreading.[42] However, whether ezrin-dependent membrane translocation of calpain is related to its potential cleaving effects on ezrin, remains to be investigated.

AMPK is a serine/threonine protein kinase that is a critical mediator of energy metabolism. In endothelial cell, it has been shown that AMPK activates eNOS phosphorylation at Ser1179, subsequently producing $NO^{\bullet}$ in absence of agonist.[26] In particular, earlier work has demonstrated an independent role of AMPK in mediating VEGF activation of eNOS via direct augmentation of eNOS$_{s1179}$ phosphorylation.[22] More recent studies indicate that AMPK is redox-sensitively activated by peroxynitrite and H_2O_2 in endothelial cells[43,44] and is responsible for shear stress activation of eNOS.[45,46] Of note, H_2O_2 activation of eNOS was found dependent on parallel activation of AKT and extracellular signal-regulated kinase 1/2.[40] It is interesting to speculate that AMPK might lie upstream of AKT activation in this cascade. In the present study, we found that AMPK activation is downstream of PI3K, which is consistent with previous observations.[44] The findings that AKT activation by VEGF is at least partially AMPK-dependent are consistent with recent reports by Levine et al.[25] In addition, AMPK-dependent AKT activation has been reported in adiponectin-induced angiogenesis,[23] and inhibition of AMPK phosphorylation by insulin-stimulated AKT activation was proposed as a cardioprotective mechanism.[24,47] Despite these controversies, our data clearly demonstrate that in endothelial cells, VEGF activation of eNOS is dependent on parallel PI3K/AMPK/AKT and PI3K/AKT pathways. What remains to be fully elucidated is whether there is a direct interaction between AMPK and AKT or, instead, whether both their direct and

indirect effects on eNOS activation occur at the same signaling domain.

Ezrin is a cytoskeletal linker protein with unclear functional roles in endothelial cell signal transduction. Our present study demonstrated an novel and critical role of ezrin in regulating membrane-specific activation of calpain and subsequent activation of eNOS. This enhances $NO^{\bullet}$ production in response to VEGF, which would be highly significant in modulating endothelial cell functions. Of note, previous studies have implicated important cytoskeletal participation in regulating eNOS expression at mRNA levels, presumably via uncharacterized actin–actin binding protein(s) complex(s).[48,49] Some cytoskeletal reorganizations have also been found redox-sensitive in endothelial cells, implying potential interactions with $NO^{\bullet}$ signaling.[29,50] It has been suggested that NADPH oxidase–derived ROS not only modulates VEGF expression but also plays a critical role in VEGF-stimulated signaling linked to endothelial cell migration, as well as neovascularization.[31,32,34,38] In view of recent advances regarding roles of ROS in early VEGF-dependent signal transduction,[35,37,38] our data seem to represent a novel mechanism whereby cytoskeletal pathways contribute to redox and $NO^{\bullet}$-mediated signaling events induced by VEGF.

In summary, new findings in the present study established a critical role of calpain/ezrin interaction in mediating VEGF-dependent signaling to eNOS via AMPK and AKT. Although detailed mechanisms underlying ezrin-dependent membrane translocation and activation of calpain, AMPK activation of AKT, as well as calpain activation of PI3K all remain to be fully investigated, our data no doubt have unraveled an important component of acute VEGF signaling at membrane level. Because ezrin and calpain are ubiquitously expressed, these findings may later prove to be broadly applicable and significant in fundamental cell biology, particularly relevant to mitogenic signaling in cardiovascular system. These data represent the first evidence that calpain signaling, via interaction with ezrin, plays a critical role in modulating endothelial cell function, in particular, mediating agonist-dependent $NO^{\bullet}$ production.

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Disclosures

None.

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