



PKC δ Regulates Force Signaling during VEGF/CXCL4 Induced Dissociation of Endothelial Tubes

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Abstract

Wound healing requires the vasculature to re-establish itself from the severed ends; endothelial cells within capillaries must detach from neighboring cells before they can migrate into the nascent wound bed to initiate angiogenesis. The dissociation of these endothelial capillaries is driven partially by platelets' release of growth factors and cytokines, particularly the chemokine CXCL4/platelet factor-4 (PF4) that increases cell-cell de-adherence. As this retraction is partly mediated by increased transcellular contractility, the protein kinase c- δ /myosin light chain-2 (PKC δ /MLC-2) signaling axis becomes a candidate mechanism to drive endothelial dissociation. We hypothesize that PKC δ activation induces contractility through MLC-2 to promote dissociation of endothelial cords after exposure to platelet-released CXCL4 and VEGF. To investigate this mechanism of contractility, endothelial cells were allowed to form cords following CXCL4 addition to perpetuate cord dissociation. In this study, CXCL4-induced dissociation was reduced by a VEGFR inhibitor (sunitinib malate) and/or PKC δ inhibition. During combined CXCL4+VEGF treatment, increased contractility mediated by MLC-2 that is dependent on PKC δ regulation. As cellular force is transmitted to focal adhesions, zyxin, a focal adhesion protein that is mechano-responsive, was upregulated after PKC δ inhibition. This study suggests that growth factor regulation of PKC δ may be involved in CXCL4-mediated dissociation of endothelial cords.

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Introduction

Wounds prevent skin from acting as a barrier between the body and external harsh environment, a vital function that must be quickly re-established during wound repair. This repair requires a healthy vascular system for both tissue generation and subsequent maintenance. Blood vessels are compromised during wounding which require angiogenesis from the tips of severed vessels for regeneration. This is driven by pro-angiogenic growth factors released first by platelets and then by macrophages in the wound. Endothelial cells that are involved in angiogenesis require initial signals to 'dedifferentiate' and separate from the existing severed vessels prior to subsequent inductive signals to migrate into the wound bed. These early wound response signals to initiate angiogenesis are mediated through platelets during clotting including the chemokine CXCL4 and growth factors VEGF, PDGF, HB-EGF, and TGF [1].

Many of the intracellular signaling pathways that drive fibroblasts and endothelial cells to migrate are known. Downstream of growth factor receptor activation, PLC γ 1 signaling triggers PKC δ to regulate cell motility via increasing transcellular contractility in fibroblasts and endothelial cells [2–8]. Growth factor and matrikine signaling through the epidermal growth factor receptor (EGFR) initiates motility via phosphorylation and activation of PLC γ 1 at the membrane [3]. Activated PLC γ 1 then catalyzes the hydrolysis of PIP₂ (primarily at the leading edge of the membrane) and generates diacylglycerol (DAG) and IP3

[9,10]. Increased levels of DAG at the leading edge [11] synergizes the effect of PKC δ to the membrane [12]. DAG subsequently stabilizes the activation of PKC δ through direct binding of its N-terminal C1 domain [13–15]. Furthermore, PKC δ localization behind the leading edge allows it to propel the cell body towards the extended lamellipodium and also mediate isometric force associated with motility [16]. It has also been previously demonstrated that downstream of PKC δ signaling, an intermediate kinase, specifically myosin light chain kinase (MLCK), relays signaling to MLC through direct phosphorylation [8]. Further supporting EGFR induced regulation of contractility, it was demonstrated that reduced activation of PLC γ 1 delayed subsequent activation of PKC δ and downstream MLC-2 [8]. These data have shown that EGFR triggers contractile responses efficiently and quickly through the PLC γ 1/PKC δ pathway.

These findings led us to ask whether such a pathway could also account for the initial retraction of severed vessels prior to angiogenic sprouting. Increased PKC δ activation has also been shown to increase vascular permeability [17–19], consistent with cell-cell de-adherence. Inhibition of PKC δ results in downregulation of stress fibers and focal adhesions in endothelial cells [20]. In addition, steady-state activation of stress fiber tension by PKC δ has been found to strengthen the endothelial cell barrier [21,22]. All of these data implicate biphasic PKC δ regulation in maintaining vascular permeability. Furthermore, MLCK that is downstream of PKC δ is involved in endothelial retraction through

MLC-2 phosphorylation. These signals initiating endothelial retraction may be essential for the initial phases of endothelial cell separation from damaged vessels prior to angiogenic sprouting. However, the mechanism in which PKC δ mediates endothelial cell retraction has not been determined.

Therefore, we hypothesized that PKC δ regulates the tension of endothelial cords by promoting cell retraction during neo-angiogenesis through VEGFR signaling in addition to CXCL4 mediated retraction.

Results

CXCL4+VEGF-induced dissociation is VEGFR-dependent

To investigate endothelial CXCL4-mediated dissociation, we used human microvascular endothelial cells (HMEC-1) plated on Matrigel. Cell cords were given 24 hours to form and then subsequently induced to dissociate with CXCL4 and VEGF, two factors released by platelets during the initial stages of hemostasis. Inhibition of VEGFR/PDGFR signaling using sunitinib (2.5 μ M) inhibited dissociation, as demonstrated by longer cord length (Fig. 1). In addition, CXCL4 had a more pronounced effect than VEGF on mediating tube dissociation (Fig 1).

VEGFR/CXCL4-induced dissociation is partially PKC δ -dependent

Tube dissociation involves the separation of cell-cell contacts, we therefore investigated whether transcellular contractility involves PKC δ , a key signaling nexus in contractility and tension [7,8]. Dissociation was blunted by downregulation of PKC δ . Formed cords were stimulated to dissociate with CXCL4 and VEGF in the presence or absence of antisense towards PKC δ .

Endothelial cord length reduction by CXCL4/VEGF was partially rescued by antisense against PKC δ (Fig. 2a). PKC δ downregulation attenuated cord dissociation to a significantly lesser degree. In addition, the antisense decreased PKC δ and MLC-2 levels that are downstream of PKC δ regulation (Fig. 2b, Fig 2c). In addition, adult primary endothelial cells were investigated, as we found that MLC-2 was also down regulated through rottlerin inhibition, a PKC δ inhibitor (Fig. 2d). In response to VEGF/CXCL4, ppMLC-2 levels in adult primary endothelial cells were increased, in contrast to CXCL4 only treatment (Fig. 2d). To further investigate endothelial cords themselves for increased contractility, HMEC-1 endothelial cord lysates were analyzed in which showed increased MLC-2 levels in response to VEGF/CXCL4 (Fig. 2e). These data indicate that PKC δ affects MLC-2 expression in a VEGF-dependent manner, which in turn correlates with its regulation of stress fibers.

Dynamics of endothelial cords/capillaries tension require PKC δ -dependent motility of cords and individual cells

Cell motility is critical for vessel formation and vessel regression [23]. To further investigate the role of PKC δ activation during VEGF/CXCL4 mediated dissociation, adult primary endothelial cords were induced to dissociate in the presence of rottlerin (500 nM). Cord motion that is a part of the process of vessel maturation was observed in both groups, however PKC δ inhibition through rottlerin reduced cord mobility (Fig 3, Sup. Movie 1,2). According to these data, dissociation was mediated by the motility of the cells and PKC δ -dependent regulation of cytoskeleton/isometric contractions.

We further investigated cord dissociation/mobility and found increased mobility and contractions in cords that were treated with

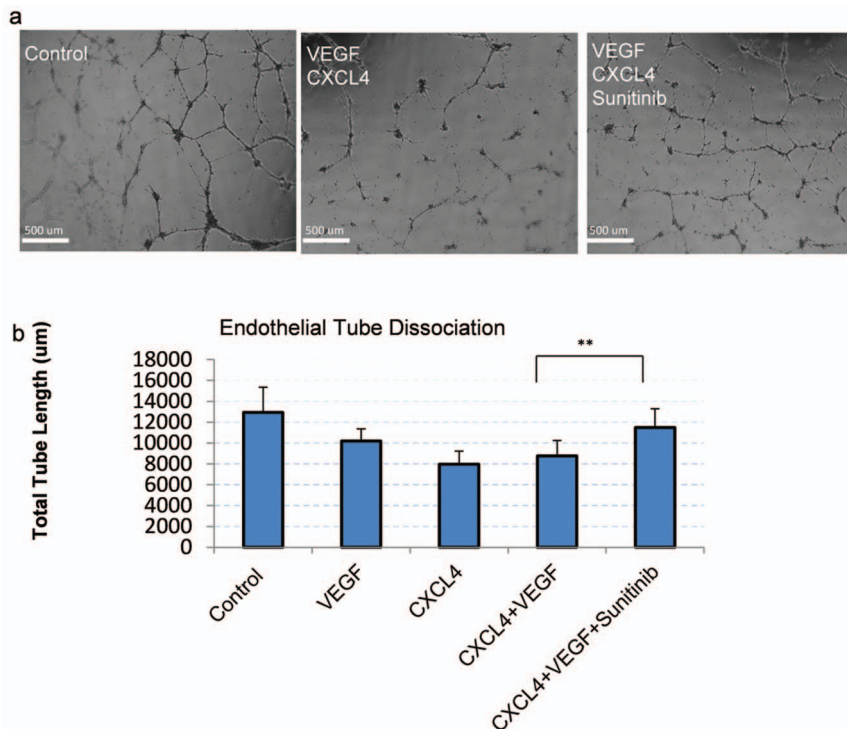


Figure 1. VEGFR inhibition decreases CXCL4-induced cord dissociation. a) Representative phase contrast images of HMEC cells treated with indicated treatments are shown. Images were taken of live cords after 24 hours. Disruption in webbed patterning of cells indicates increased dissociation. b) Images described in (Fig 1a) were quantified as described in methods using Metamorph analysis software (N at least 4; mean \pm SD, ** P<0.05), Shown are representative of 3 experiments.

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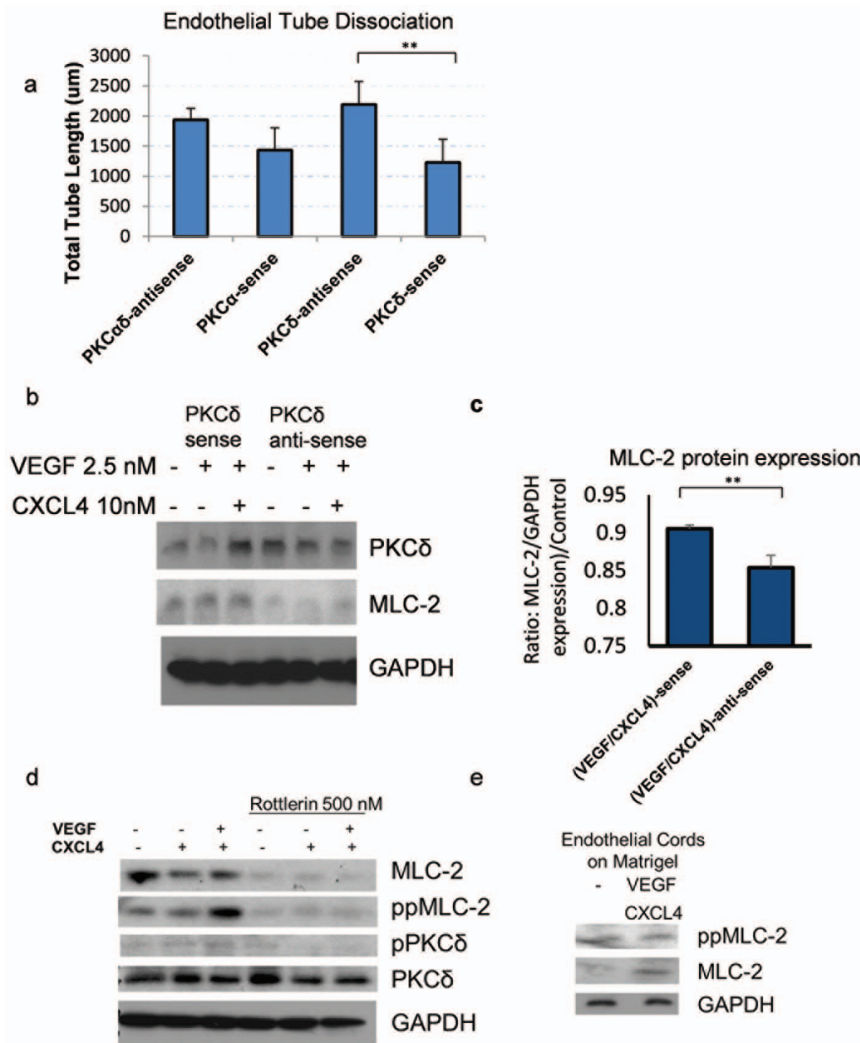


Figure 2. PKC δ inhibition decreases CXCL4-induced cord dissociation. a) Cords that were induced to dissociate were quantified using Metamorph as in (Fig. 1b). HMEC cells were allowed to form cords. Following 24 hours, 20 μ M of antisense/sense oligonucleotides were added to cords as described in methods and allowed to dissociate with inhibition of PKC δ for 24 hours. Cords and cord length were measured (N at least 4; mean \pm SD ** $P < 0.05$); blots shown are representative of 3 experiments. b) Immunoblot analysis of HMEC monolayer lysates during a 24 hour addition of CXCL4 (10 nM) and VEGF (2.5 nM) in the presence of antisense inhibition. GAPDH was used as a loading control. c) MLC expression was analyzed from (Fig 2b) with either PKC δ antisense or sense in response to CXCL4 (10 nM) and VEGF (2.5 nM) (N=3; mean \pm SD ** $P < 0.05$). d) Primary adult dermal human microvascular endothelial cells were treated with indicated CXCL4 (10 nM) and VEGF (2.5 nM) in the presence and absence of PKC δ inhibitor (Rottlerin 500 nM) for 24 hours. Cell lysates were analyzed using immunoblot analysis. GAPDH was used as a loading control, as the control representative of the two blots shown. e) HMEC-1 cells were plated on Matrigel to form cords in a 6-well plate. After cords were formed, cells were treated with CXCL4 (10 nM) and VEGF (2.5 nM) for 24 hours. Western blot analysis was performed on lysates of both endothelial cords and Matrigel. GAPDH was used as a loading control.
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CXCL4/VEGF (Movies S3, S4). In addition, increased force was exerted onto the Matrigel by the endothelial cells, as shown by the deformation of the substratum noted in live cell imaging via phase contrast microscopy. Individual endothelial cells at their junctions were compressed and spheroid as this morphology appeared to enable the necessary movements for increased mobility for subsequent cord collapse (Movie S4).

Activation of PKC δ is increased during CXCL4/VEGF-induced dissociation of endothelial cords

To further investigate whether PKC δ was activated during dissociation, levels of PKC δ and activated (or phosphorylated) PKC δ were measured in endothelial cords. In CXCL4+VEGF-

induced cells, this ratio was increased at junctions with increased phosphorylated PKC δ at junction edges and decreased PKC δ in the inner part of the junction (Fig. 4a). In addition, in CXCL4 only- and CXCL4+VEGF-mediated dissociation, endothelial cells with a spheroid morphology and increased levels of activated PKC δ were also observed. Although PKC δ has been shown in previous literature to mediate apoptosis [24,25], it seems that these cells may actually alter their mesenchymal phenotype to incorporate into the cords and to actively mediate tension at junctions (Fig 4b, Movie S4). This would be consistent with our finding that PKC δ directing cell contractility [7]. These data imply that activation of PKC δ is associated with force generation and possibly force mechanics in endothelial cord dissociation.

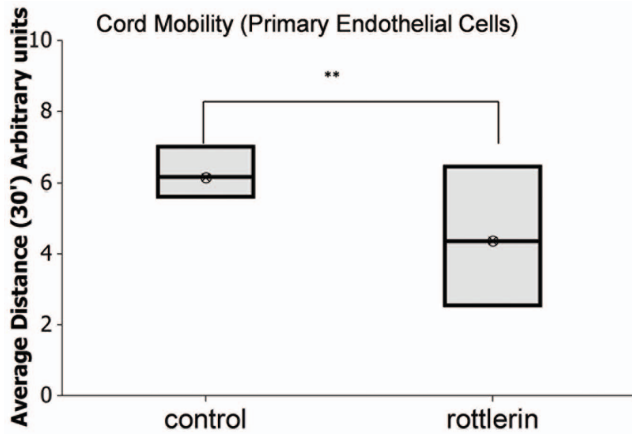


Figure 3. PKC δ inhibition limits the mobilization/tension on the cords. Quantification of cord motion in supplemental movies 1 and 2. Live cell imaging of cords as they move were quantified with metamorph analysis (Sup. Movie 1/2), as each cord excluding branches were tracked and quantified as average distance displacement for 30' (N = 14 cords measured; mean \pm SD $^{**}P < 0.01$). doi:10.1371/journal.pone.0093968.g003

Mechanotransduction of VEGF/CXCL4 induced cord dissociation is PKC δ -dependent

Cord dissociation involves multiple intercellular forces which coordinate the subsequent collapse of capillaries. To confirm that force impacts the endothelial cords molecularly, focal adhesions/junctions were examined as cellular locations affected by CXCL4/VEGF induced dissociation. As a focal adhesion protein, the zyxin protein has been previously found regulated in response to vascular stretch. In this study, zyxin was used to investigate mechanosensory input into the cords as they dissociate. The investigation of zyxin showed that its expression is linked to PKC δ activity, as inhibition of PKC δ caused increased zyxin expression in 2D monolayer (Fig 5a). Furthermore, downregulation of PKC δ with antisense oligonucleotides in formed endothelial cords led to increased zyxin expression (Fig 5b). These data suggest focal adhesions are dynamically regulated when force is induced. Furthermore, PKC δ that is inhibited by a dominant negative construct causes focal adhesions to stabilize, thus contributing to inhibition of cord dissociation.

These data suggest that force signaling through VEGFR is applied during cord dissociation through motility signaling via PKC δ . Signaling through VEGFR causes PKC δ to elicit both static and dissociative regulation of force for endothelial capillary stability and dissociation, respectively.

Discussion

The CXCL4 receptor, CXCR3, primarily regulates endothelial capillary dissociation through calcium regulation. When calcium channels are stretch-activated, u-calpain is activated that results in cleavage of focal adhesions leading to cell de-adherence and subsequent anoikis/apoptosis [26]. In this study, we investigated VEGFR signaling to PKC δ as a non-direct molecular mediator of CXCL4-induced cord/capillary dissociation. Based on the results of this study, PKC δ that is activated downstream of VEGFR tyrosine kinase signaling plays a role in endothelial cell dissociation (Fig. 1). Attenuation of cord dissociation through inhibition of VEGFR and PKC δ indicates that, for full dissociation of cords on Matrigel, VEGFR signaling through PKC δ is required (Fig. 1a, Fig. 1b). VEGFR regulation also mediates increased regulation of

PKC δ , as seen in our data when PKC δ levels were upregulated (Fig. 1d). PKC δ regulation of contractility through MLC-2 was observed in data where PKC δ antisense and PKC α antisense caused MLC-2 downregulation after CXCL4-VEGF treatment (Fig. 1d). However, the effect of PKC α antisense was lower, and may affect PKC δ activity indirectly through an unknown mechanism. We further inhibited the dissociation response using the pharmacological agent rottlerin and by causing the cell cords to become static and non-motile (Movie S1, S2, Fig. 3). Since motility is essential for cord formation, our data also highlight the importance of cord integrity (Movie S3), which translates to cord retraction and dissociation (Movie S4).

PKC δ activation was also investigated during cord dissociation. The ratio of activated PKC δ to PKC δ was increased at junctions in CXCL4-VEGF-treated cells (Fig. 4). Moreover, some cells that were activated with PKC δ were circular and were centrally located within junctions (Fig 4). It is interesting to consider whether these cells are functionally important in integrating cord force as the cords dissociate or move. Further experiments are needed to fully test this hypothesis. If further investigation supports this idea, it would offer new insight into how PKC δ regulates motility and force in microvasculature.

We further showed that cord motility and dissociation are directly linked to PKC δ regulation. PKC δ seems to contribute to dissociation more by affecting the plasticity of active movement than by disrupting intracellular junctions mediated by calpains (Fig 1). In this investigation, we show that PKC δ regulates dissociation through VEGFR to PLC γ 1 signaling. However, it remains unknown whether force signaling is directing the dissociation, or recursively responding to force regulation. Both situations require PKC δ , but in a different context. These results also demonstrate that motility mechanisms seen in 2D migration play an important role in multi-cellular tissue. Increased calcium signaling perpetuates increased contraction and de-adhesion, while VEGFR signaling is also mediating and directing contractions through PKC δ signaling.

After dissociation has occurred, stabilization of PKC δ may still mediate further contraction of cords in the right context [21,22,27]. As cords shift to a more stabilized and quiescent cellular state, signaling from VEGFR and other receptors with intrinsic tyrosine kinase activity continues in order to further activate downstream mediators of PKC δ . These events fine-tune the development of a normal vascular structure and help stabilize autocrine signaling. Alternately, angiogenic driven VEGF stimulation induces upregulation of PKC δ that would result in destabilization of the vasculature, with the separated endothelial cells now being responsive to motile signaling. This is combined with extracellular signaling of other molecular mediators and regulation by the stroma, allowing for remodeling of the wound bed. Lastly, pericytes also regulate the microvasculature with force regulation [28,29]. Studies investigating how pericytes regulate cord stability/dissociation would further elucidate how force signaling is modulated *in vivo*. Such studies represent the next step in discerning the function of cord mobility in vascular regeneration.

Methods

HMEC-1 cell culture

HMEC-1 (human dermal microvascular endothelial cells) were obtained from CDC, Atlanta, Georgia, originally described by Ades et al [30]. Endothelial cells were grown in MCDB 131 (Gibco) media with 10 mM L-glutamine supplemented with 10% fetal bovine serum. For stable expression of the zyxin protein,

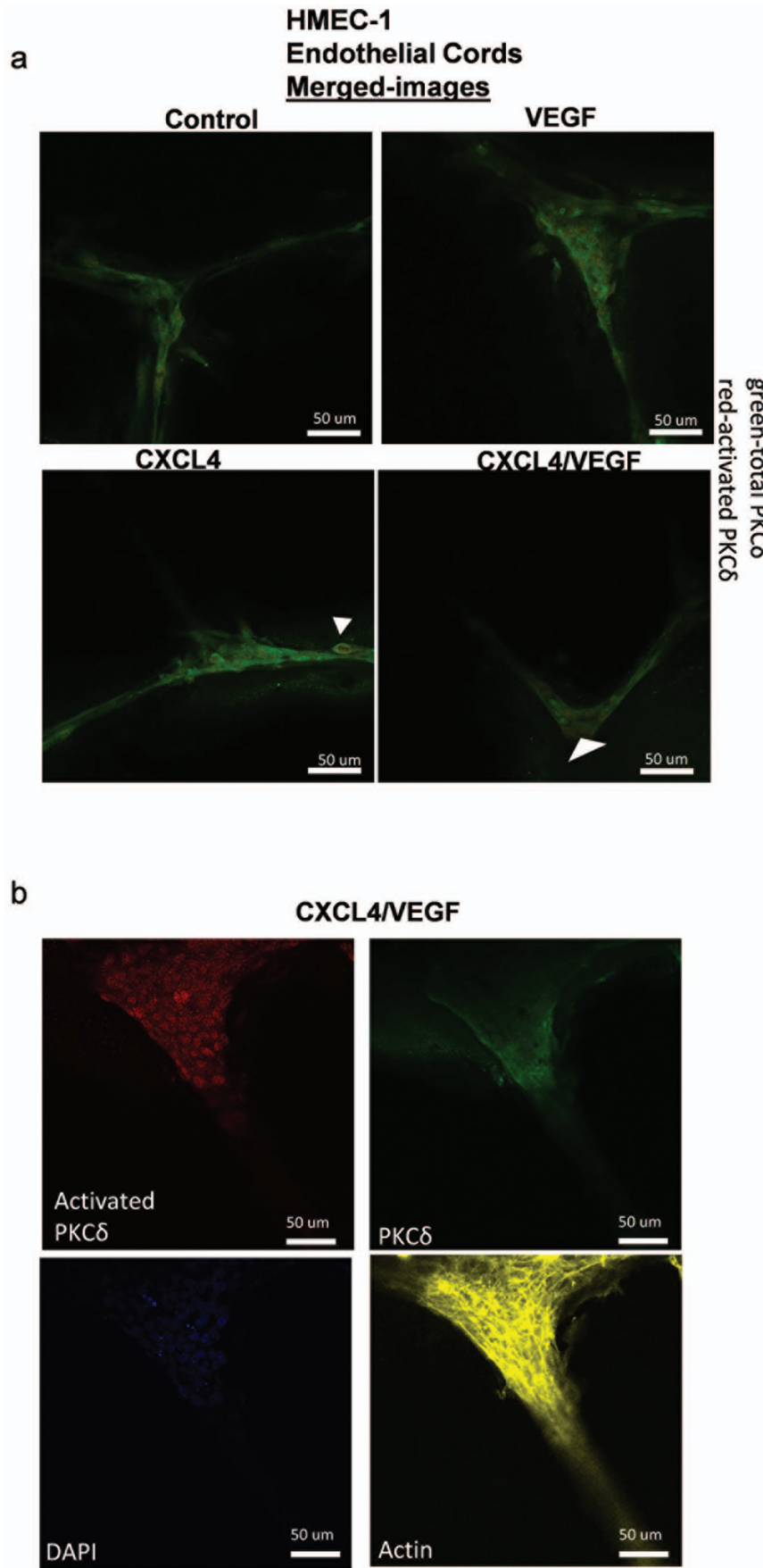


Figure 4. PKC δ is activated during cord dissociation. a/b) Immunostaining of HMEC endothelial cells formed into cords onto Matrigel treated with indicated treatments for 24 hours after cords are formed. a) Merged representative images of activated PKC δ (red) and PKC δ (green) for indicated treatments. b) Representative image of CXCL4 100 nM/VEGF 2.5 nM treated cord that was immunostained with PKC δ and phospho-PKC δ indicated in green and orange separate images. Cells were also stained for nuclei with DAPI staining represented as blue and actin with phalloidin staining (Alexa-633) represented in pseudo-color yellow. Representative image of a larger cord indicates increased effects of force translated in the ratio of activated PKC δ to PKC δ . Arrows of increased phosphorylated PKC δ expressing cells indicates positioning of possibly active cells at branch points of cords.
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HMEC-1 cells were transfected with 4 μ g of GFP-Zyxin plasmid (obtained from Origene) with 10 μ L of lipofectamine in 6 well dish with subconfluent cells. Stable selection of Zyxin focal adhesion markers in HMEC-1 cells was established with neomycin selection (350 ng/mL) as cells were passaged.

Adult-Dermal Human Microvascular Endothelial Cell culture

Primary human endothelial cells were obtained from (Life Technologies) and were grown in EGM-2MV Media, (Lonza). Cells were grown 5 passages prior to incubating cells in quiescent media for 24 hour before experiment.

Matrigel tube formation assay

Cells were grown to 50% confluency prior to seeding onto Matrigel. Growth Factor Reduced (GFR) Matrigel (BD Biosciences) was seeded onto a u-chamber slide at 10 μ L per well or 120 μ L per well on a 24-well plate and incubated for 30 minutes at 37°C for polymerization. Cells were then re-plated onto Matrigel at 75,000 cells per well (on the 24-well plate) or 15,000 cells per well (on the u-chamber slide, Ibidi) for 24 hours. Cords were grown overnight in quiescent media 0.5% FBS. Afterwards, endothelial cords were allowed to dissociate with VEGF-165 Recombinant growth factor and Recombinant CXCL4 (Pepro-Tech), which were added at 2.5 nM and 100 nM respectively after 24 hours (or as indicated by the time points given in figure/movie). Some experiments were quantified for cord length by adding 2.5 μ M Cell Tracker Green for better quantification.

Antisense inhibition

DNA oligonucleotides sequences targeting PKC δ and PKC α were obtained from the literature [31,32] and ordered from IDT; phospho-thioate bonds were added to the end nucleotides. Oligonucleotides sense and anti-sense of human PKC δ are GTGGCATGATGGAGCCTTTT and 5'TTTTCCGAGG-TAGTACCGTG-3', respectively. Oligonucleotide sense and antisense PKC α are 5'-CGGGCAACGACTCCACGGCG-3' and '5-CGC CGT GGA GTC GTT GCC CG-3', respectively. DNA oligonucleotides at 20 μ M were added after HMEC cords were formed in quiescence media, and additional antisense was added to the cells via lipofectamine (10 μ L) for 0.5 μ g of DNA in 500 μ L of OPTI medium per sample in 6 well plate. DNA/Lipofectamine was added to quiescent media overnight prior to growth factor/cytokine treatment.

Live cell imaging

HMEC-1/Primary endothelial cells were grown and plated in a 6 well glass bottom plate on Matrigel at 75,000 cells per well and incubated in quiescent media overnight. Endothelial cords that formed were treated with indicated treatments and immediately added to live cell chamber with 5% O₂, 5% CO₂, or 90% N₂ at 37°C. Primary endothelial cells (Fig. 3, Movie S1, Movie S2) were imaged for 7 hours and HMEC-1 cells were imaged for 1 hour and 20 minutes at 5 minute intervals (Movie S3 and Movie S4). Images were taken of endothelial cords on top of Matrigel with a Nikon Eclipse Ti live cell imaging microscope at 10 \times and 20 \times objective respectively as size bars are indicated in live cell imaging movies.

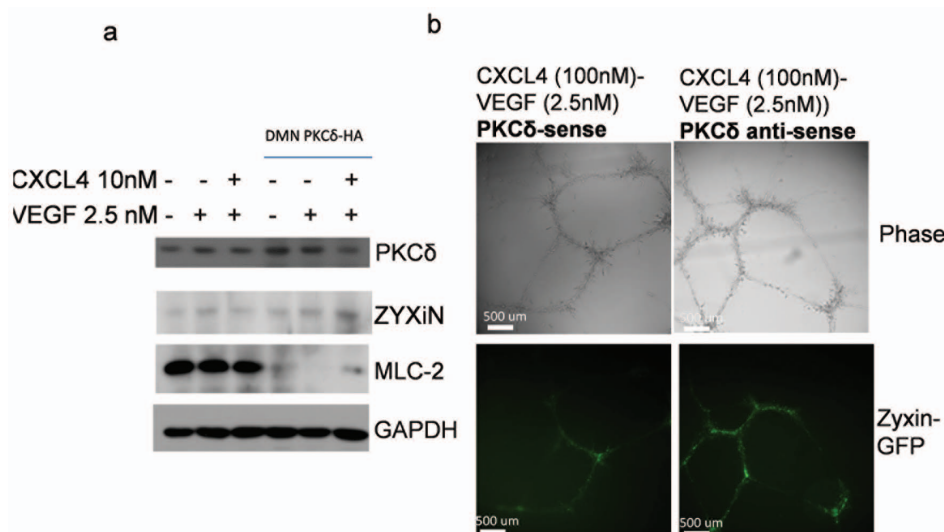


Figure 5. The tension-sensor zyxin is elevated in the absence of PKC δ . a) Immunoblot of HMEC monolayer transfected with Dominant negative PKC δ DNA vector (4 μ g/lipofectamine[10 μ l]) prior to treatment with CXCL4/VEGF in quiescence media for 24 hours. b) Live cell fluorescent images of Zyxin-expressing HMEC cells that have formed cords. After cords were formed, cells were incubated for 5 days with 20 μ M PKC δ -sense or PKC δ -anti-sense in VEGF 2.5 nM/CXCL4 100 nM. Shown are representative of 3 experiments.
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Immunostaining/confocal microscopy

HMEC-1 cells cultured and plated onto 15 well u-chamber slides (Ibidi) as previously described. After selected treatments, cells were fixed in 2% formaldehyde in PBS for 10 minutes. Cells were then permeabilized with 0.1% Triton-X-100 in PBS (wash buffer) for 10 minutes. Afterwards, cells were washed for 30 minutes (3× washes) prior to incubation with antibodies. Antibodies were diluted (1:50) for anti-phospho-PKC- θ/δ (S643/S676)(Cell Signaling) polyclonal rabbit and (1:100) anti-PKC δ (polyclonal anti-mouse) in wash buffer with 30 mg of bovine serum albumin. Cells were immunostained overnight at 4°C. Cells were then washed for 15 minutes (3×) and incubated with secondary antibody at (1:100) (Alexa-488-antimouse and Alexa-594-antirabbit- LifeTechnologies) in wash buffer/30 mg of BSA/5% goat serum, as secondary antibodies were goat. Antibodies were incubated onto cells for 1 hour at room temperature before cells were washed (3×). Alexafluor-633 phalloidin (1:40) in PBS and DAPI (1:10000) was added to cells. Afterwards, cells were desiccated for 60' at RT to decrease matrigel depth (z) above 180 μm in micro-chamber slide. PBS was added to cells for 30 minutes prior to imaging and were imaged on an Olympus Fluoview FV1000 confocal IX81 microscope.

Metamorph analysis

Total cord length was measured using Metamorph analysis software. Minimum cord length was set at 1 μm or 1 pixel and max cord width was set to 30 μm . The track object function in metamorph was used to track cord motion.

Statistical Analysis

One-way ANOVA was performed (Fig. 1) with significance being determined at $p < 0.05$. Two sample Student's T test was performed for all other experiments with significance being determined at $p < 0.05$.

Supporting Information

Movie S1 Representative movie of adult primary dermal human microvascular endothelial cords in quiescent media. After cords were formed rottlerin (500 nM) was added to group (Movie S2) immediately before imaging. Cell

tracker orange was added at 2.5 μM concentration for better observation of groups immediately before imaging. Endothelial cords were imaged 7 hours for 30 minutes per frame, as movie represents merged images of red fluorescence and phase. Scale bar of 50 μm is indicated on lower right hand corner; images were taken at 10× objective lens.

(AVI)

Movie S2 Representative movie of adult primary dermal human microvascular endothelial cords in quiescent media. After cords were formed rottlerin (500 nM) was added to group (Movie S2) immediately before imaging. Cell tracker orange was added at 2.5 μM concentration for better observation of groups immediately before imaging. Endothelial cords were imaged 7 hours for 30 minutes per frame, as movie represents merged images of red fluorescence and phase. Scale bar of 50 μm is indicated on lower right hand corner; images were taken at 10× objective lens.

(AVI)

Movie S3 Representative movies of HMEC-1 endothelial cords were taken in either quiescent media or with CXCL4 (100 nM)/VEGF (2.5 nM) added immediately before imaging (Movie S4). Phase contrast images were taken for a duration of 1 hour 20 minutes at 5 minutes per frame. Scale bar of 50 μm is indicated.

(AVI)

Movie S4 Representative movies of HMEC-1 endothelial cords were taken in either quiescent media or with CXCL4 (100 nM)/VEGF (2.5 nM) added immediately before imaging (Movie S4). Phase contrast images were taken for a duration of 1 hour 20 minutes at 5 minutes per frame. Scale bar of 50 μm is indicated on lower right hand corner; images were taken at 20× objective lens.

(AVI)

Author Contributions

Conceived and designed the experiments: JJ JW AW. Performed the experiments: JJ. Analyzed the data: JJ JW AW. Contributed reagents/materials/analysis tools: JW. Wrote the paper: JJ AW. N/A.

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