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Endothelial cell calpain as a critical modulator of angiogenesis

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Abstract

Calpains are a family of calcium-dependent non-lysosomal cysteine proteases. In particular, calpains residing in the endothelial cells play important roles in angiogenesis. It has been shown that calpain activity can be increased in endothelial cells by growth factors, primarily vascular endothelial growth factor (VEGF). VEGF/VEGFR2 induces calpain 2 dependent activation of PI3K/AMPK/Akt/eNOS pathway, and consequent nitric oxide production and physiological angiogenesis. Under pathological conditions such as tumor angiogenesis, endothelial calpains can be activated by hypoxia. This review focuses on the molecular regulatory mechanisms of calpain activation, and the newly identified mechanistic roles and downstream signaling events of calpains in physiological angiogenesis, and in the conditions of pathological tumor angiogenesis and diabetic wound healing, as well as retinopathy and atherosclerosis that are also associated with an increase in calpain activity. Further discussed include the differential strategies of modulating angiogenesis through manipulating calpain expression/activity in different pathological settings. Targeted limitation of angiogenesis in cancer and targeted promotion of angiogenesis in diabetic wound healing via modulations of calpains and calpain-dependent signaling mechanisms are of significant translational potential. Emerging strategies of tissue-specific targeting, environment-dependent targeting, and genome-targeted editing may turn out to be effective regimens for targeted manipulation of angiogenesis through calpain pathways, for differential treatments including both attenuation of tumor angiogenesis and potentiation of diabetic angiogenesis.

Keywords

Angiogenesis; Calpain; Endothelial cell; VEGF; eNOS; Tumor angiogenesis; Diabetic wound healing; Atherosclerosis; Retinopathy; Shear stress

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1. Introduction of calpains

Calpains belong to a family of calcium-dependent cysteine proteases. Calpains proteolytically process substrates to transform their structures to modulate activities. Calpains have been present during evolution. Isoforms of calpains exist in various organisms, ranging from fish to human [1–3]. To date, 15 isoforms have been identified in humans [4]. Calpain 3 is found in skeletal muscle, while calpain 6 in placenta and embryonic muscles, calpain 8 and calpain 9 in gastrointestinal tract, calpain 11 in testis and calpain 12 in hair follicles. Other calpain isoforms are ubiquitously expressed. Among all of the calpain isoforms, calpain 1 and calpain 2 have been widely studied. Of note, calpain 1 and calpain 2 are the only known calpain isoforms expressed in the endothelial cells [5]. Calpain 1 (p-calpain) and calpain 2 (m-calpain) were originally named by μmol or mmol of calcium concentration that is required for their activation in vitro. The requirements for half maximal activities are at approximately 3–50 μM and 400–800 μM for calpain 1 and calpain 2 respectively [6,7]. However, additional evidences indicate that both calpains could be activated at physiological calcium concentrations in vivo at 0.4–0.5 μM (calpain 1) and 10 μM (calpain 2) respectively, although the activation mechanisms may involve specific cellular components, such as membrane ezrin and phosphoinositides [8–14].

The calpain proteolytic system includes large subunits, small subunit and the endogenous calpain inhibitor calpastatin. Calpain 1 (CAPN1) and calpain 2 (CAPN2) are heterodimeric proteins, consisting of a large 78–80 kDa catalytic subunit and a common 29 kDa small regulatory subunit (CAPN4). Calpastatin has been shown to inhibit both calpain 1 and calpain 2. Upon activation, calpains cleave a broad spectrum of substrates that are involved in several fundamental cellular processes, including cell proliferation, cell migration and cytoskeletal remodeling. Several groups have shown that calpains are required for cell proliferation. Inhibition of calpains leads to reduced proliferation in different cell types, such as pulmonary artery smooth muscle cells, HeLa and WI-38 human fibroblasts [15–17]. It is also reported that calpain-mediated substrate proteolysis is indispensable for cell migration and cytoskeletal remodeling [18,19]. Attenuated cell migration was observed in cells treated with calpain inhibitors [20,21]. Calpain-mediated proteolysis of focal adhesion proteins, such as paxillin, focal adhesion kinase and talin, mediates focal adhesion turnover during migration [22–24]. Mouse embryonic fibroblasts obtained from CAPN4 knockout mice displayed repressed migration and impaired organization of cytoskeleton [25]. In this review, we will discuss the mechanistic roles and downstream signaling events of endothelial calpains in both physiological and pathological angiogenesis.

2. The structure of calpains

The catalytic large subunit of calpains comprises four domains of I-IV, while the small subunit has two domains V-VI. The N-terminus of domain I undergoes autolysis upon exposure to Ca^{2+} [26,27]. Domain II contains the catalytic sequence of Cys-His-Asn. The crystallographic structure of calpain reveals that in the absence of calcium the active site is disrupted by the separation of the two subdomains, domains Ha and lib [8,28]. Upon Ca^{2+} binding, however, lib and Ha form a functional catalytic center following a subtle conformational change [8,28]. The crystal structure of calpain 2 similarly reveals that during the conformational change, some residues in lib come into close contact with the membrane [8]. Domain III can bind to phospholipid in Ca^{2+} - dependent manner, and may play an important role in Ca^{2+} -dependent membrane translocation of calpains [29,30]. We have shown that Ezrin is required for the membrane specific activation of calpain [14]. Domain IV and domain VI each contains five EF-hand motifs. It has been shown that the first to third EF-hands bind Ca^{2+} and the fifth EF-hand motif is involved in heterodimer formation of the large subunit and the small subunit. Domain IV and domain VI are also involved in binding to calpastatin [31]. Domain V of the regulatory small subunit contains hydrophobic Gly-rich sequence [32,33]. Deletion of domain V reduced membrane binding and localization [34]. Most of this domain is cut off by autolysis, indicating no involvement in protease activity.

3. Activation of calpains in endothelial cells

Activation of calpains in endothelial cells is induced by growth factors, primarily vascular endothelial growth factor (VEGF). It has been reported that VEGF activates calpains in endothelial cells, which can be attenuated by calpain inhibitors calpeptin, ALLN and calpastatin [14,35,36]. The detailed mechanisms of calpain activation by VEGF are discussed below. The combination of VEGF and basic fibroblast growth factor (FGF) was reported to significantly elevate calpain activity in endothelial cells, as well as the cleavage of calpain substrate vimentin [37]. Another growth factor that has been shown to activate calpain 2 in endothelial cells is epidermal growth factor (EGF). It has been recently reported that EGF induces calpain 2 membrane translocation and activation in endothelial cells through phosphatidylinositol 4,5-bisphosphate [38]. Nonetheless, VEGF seems to be the primarily characterized activator of calpain signaling in endothelial cells.

Under physiological conditions, activation of calpain by VEGF results in endothelial nitric oxide synthase (eNOS) phosphorylation to produce nitric oxide (NO), which is a potent angiogenic activator [14]. It is known that VEGF activates eNOS/NO through PI3K/Akt [39–41]. Our group previously identified a novel role of calpain 2 in mediating VEGF-induced PI3K/AMPK/Akt activation, and subsequent eNOS phosphorylation and NO production in endothelial cells [14]. Application of calpain inhibitors (ALLN or Calpeptin), or siRNA targeting calpain 2, abolished VEGF-induced activation of PI3K/AMPK/Akt/eNOS/NO pathway. However, calpain 1 is not involved in this response [14]. Taken together, these data indicate a unique role of calpain 2 in mediating VEGF-induced angiogenesis. Other reports also support the specific role of calpain 2 in mediating VEGF downstream signaling [42]. It has been shown that VEGF selectively activates calpain 2 in endothelial cells [42]. The activation of calpain 2 by VEGF may be caused by preferably increased calpain 2 protein expression. Su et al. demonstrated that the protein abundance of calpain 2 was increased 2 h post-VEGF stimulation [43]. However, calpain 1 expression was unchanged, suggesting that elevated calpain activity is attributed to increased calpain 2 protein content [43,44]. Our group has further revealed a calpain dependent negative feedback loop to inhibit VEGFR2 overactivation [45]. Cleavage and activation of protein tyrosine phosphatase type 1 (PTP1B) by calpain de-phosphorylates VEGFR2 [45]. Altered expression or activity of PTP1B and/or calpain modulated VEGF-induced angiogenesis and diabetic wound healing in mice [45]. Illustrations on a role of endothelial calpain 2 in

VEGF-induced angiogenesis are presented in Fig. 1. Roles of calpain pathways in physiological and pathological angiogenesis are further discussed in the Section 4 below.

Moreover, calpains are activated when endothelial cells are exposed to hypoxia [46–49]. Zhang et al. reported that short exposure of endothelial cells to hypoxia (1–12 h) up-regulated mRNA level and activity of calpain [47]. Treatments of actinomycin D (a transcriptional inhibitor) and ALLN prevented hypoxia-induced calpain 2 transcription and activation, implicating a potential regulation at transcriptional level [47]. Though VEGF is transcriptionally regulated by hypoxia inducible factor-la (HIF-la) during hypoxia, the transcription regulation of calpain mRNA may be facilitated directly by hypoxia in short exposure. For longer exposure to hypoxia, calpain activation could be mediated by HIF-la-induced VEGF or Na^+/H^+ exchanger-1 (NHE1) expression [49]. Similar to VEGF, NHE1 is also a HIF-1α target. Both mRNA and protein levels of NHE1 are up-regulated by hypoxia (48 h) or adenovirus-delivered HIF-la (24 h) [49,50]. Knockdown of NHE1 selectively inhibited HIF-la-induced calpain 2 protein expression and activation, and subsequent angiogenesis [49]. These pathways are included in Fig. 2 for mechanisms of tumor angiogenesis involving calpain activation.

In addition to selective induction of calpain 2 expression, calpain activation is subjected to spatial regulations. We have shown that in response to VEGF stimulation, only the membrane-localized calpain is activated and inhibited by calpain inhibitors (ALLN and calpeptin). Furthermore, we also found that VEGF induces direct binding of calpain and ezrin [14]. Membrane colocalization of these two proteins can be detected within 10 min of VEGF stimulation. The activity of calpain in the membrane fraction was greatly decreased by ezrin siRNA [14]. These results suggest that ezrin mediates calpain membrane translocalization and activation as a novel mechanism of regulating calpain in endothelial cells. Another mechanism of calpain membrane activation involves phosphoinositides. Studies from different groups have shown that phosphoinositides, components of the membrane, interact with calpain 1 and 2 through calpain domain III [10,30,51]. Sphingosine 1-phosphate (SIP) induces calpain membrane translocation and activation without changing calpain expression level [52]. More evidence of calpain membrane localization was shown by the study of the crystal structure of calpain 2 [8].

Furthermore, calpain activation in endothelial cells involves calpastatin, the endogenous calpain inhibitor. It was reported that calpastatin binds to calpains in response to calcium to prevent calpains from activation [53]. Calpastatin has four inhibitory domains and each one of those is able to bind to one calpain molecule. Each inhibitory domain contains three subdomains, A, B and C. Subdomains A and C bind to calpain domain IV and domain VI, respectively [31,54]. A recent study has shown that calpastatin binds to the active cleft of calpains by looping out around the active site, so that calpastatin blocks the active site without being cleaved [54]. Treatment of endothelial cells with VEGF potently down-regulated calpastatin expression, leading to increased calpain activity [44]. The combined regulations of calpastatin and calpain enable maximal activation of calpain and its downstream signaling in response to VEGF.

Therefore, calpain 2 activation in endothelial cells can be induced through at least three different mechanisms: 1) selective up-regulation of calpain 2 protein abundance and activity [14,43,55]; 2) spatial regulation of calpain subcellular localization to promote membrane translocation and activation [14,56]; and 3) down-regulation of calpain inhibitor calpastatin [44]. The mechanistic pathways of calpain 2 activation by VEGF to mediate angiogenesis are summarized in Fig. 1. Calpain 2 inhibition suppresses multiple features of angiogenesis, such as proliferation [56,57], cell migration [42,55,58] and tube formation [42,43,55,58].

4. Role of calpains in angiogenesis

4.1. General introduction of angiogenesis

Angiogenesis is the process of new vessel formation from existing vessels. Angiogenesis requires a highly coordinated series of events that involve the interactions among endothelial cells, extra-cellular matrix and growth factors. Key steps of angiogenesis include endothelial cell proliferation, migration and tube formation (the formation of capillary-like tube structures). Basic physiology of angiogenesis has been discussed in several recent reviews [59–61]. VEGF is one of the main initiators of angiogenesis. We and others have shown that VEGF exposure induces calpain 2 dependent activation of PI3K/AMPK/Akt/ eNOS pathway and NO production in endothelial cells through VEGFR2 [14,45]. Blocking PI3K/ AMPK/Akt by pharmacological kinase inhibitors, or genetic/pharmacological abrogation of calpain 2 activation, completely attenuated VEGF-induced NO production and angiogenesis [14,62–64]. Additionally, angiogenesis is promoted in conditions where ERK is activated. Studies have shown that ERK is activated in response to VEGF/VEGFR2 [65]. Abrogated ERK signaling inhibits VEGF-induced angiogenic response of endothelial cells [66].

4.2. Role of calpains in angiogenesis under physiological conditions

Under physiological conditions, angiogenesis mostly occurs during embryonic development, when it requires adequate vasculature for organ development. Normal angiogenesis in the adulthood usually happens during repair processes, such as wound healing. It has been reported that deletion of the small subunit (CAPN4) of calpain results in elimination of both calpain 1 and calpain 2 activities and embryonic death at day Ell.5, implicating a role of calpain in embryonic development [67]. Arthur et al. reported that CAPN4 knockout embryos had reduced yolk sac vasculature at E10.5. The endothelial cells lining the atria were found rounding up at E10.5, and eventually delaminated at El 1.5, indicating the indispensable role of calpains in normal vascular development [67]. Using global and conditional knockout strategies, Takano and colleagues have shown that calpain 2 deficiencies caused embryonic death on day 15, due to placental dysfunction-induced apoptosis [68]. Interestingly, calpain 1 knockout alone appeared mostly harmless (viable and fertile) [69]. Double knockout of calpain 1 and calpain 2 potentiated embryonic lethality (— 3 days earlier) however, suggesting that the two isoforms may additively modulate some common developmental pathways in vivo, at least during the developmental stage [68].

Calpains play important roles in wound healing, a physiological process highly dependent on angiogenesis. It has been shown that transgenic mice globally over-expressing calpastatin displayed a striking delay in skin wound healing through impaired angiogenesis, indicating that calpain activity is required for wound healing [70]. We have recently demonstrated that application of calpain inhibitor ALLN to wound bed significantly delayed VEGF-induced wound healing in diabetic mice [45]. However, wound healing is not a process that only involves enhanced angiogenesis. In the late stage of wound healing, a mechanism of vessel dissociation/regression is activated. Interestingly, vessel regression is mediated by calpain 1. Bodnar and colleagues reported that activation of calpain 1 in endothelial cells leads to vessel regression and reduced angiogenesis during middle and late stage of wound healing [71,72]. Calpain 1-induced dissociation of newly formed vessels is a mechanism to maintain a regular vascular network near the wounded area by eliminating excessive vessels. Moreover, a separate study reported that cells with calpain 1 siRNA transfection showed stabilized tube formation at late stages (6–24 h) [58]. Of note, the role of calpain 1 during later stage of angiogenesis is established in experiments of exposing endothelial cells to CXCL10 (IP-10), a known ligand for the resolving stage of the wound, rather than VEGF. A potential role of calpain 1 in VEGF-induced wound healing remains to be investigated.

4.3. Role of calpains in angiogenesis under pathological conditions

4.3.1. Calpains in tumor angiogenesis

The constant growth of solid tumor requires large quantity of oxygen and nutrients. Therefore, tumor cells have developed the ability to establish their own blood supply by the induction of angiogenesis. By secreting angiogenic factors, tumor cells induce angiogenesis around them to achieve nutrient delivery and removal of metabolic wastes through the newly formed vessels. One of the most important angiogenic factors produced by tumor cells is VEGF. Blockade of VEGF or VEGF receptors dramatically inhibited tumor growth via abrogation of angiogenesis [73]. As discussed in the previous section, VEGF activates calpain 2 in endothelial cells [14,42,43,55]. We and others have demonstrated that inhibition of calpain 2 (with siRNA or calpain inhibitors) abolished VEGF-induced endothelial NO production and angiogenesis [14,43,45,74]. Moreover, the fast growth of tumor cell results in hypoxia, exposure to which upregulates calpain expression and activity in endothelial cells [46–49,75,76].

Under hypoxic conditions, calpains are involved in the crosstalk between tumor cells and endothelial cells. VEGF is secreted by tumor cells to influence endothelial cells. Interestingly, calpain in tumor cells serves as a newly identified regulator of the HIF-la/VEGF pathway [77]. Zheng et al. have shown that hypoxia induces filamin A proteolysis by calpain in melanoma cells, which in turn facilitates HIF-1α nuclear translocation. Calpeptin inhibition of calpain however attenuated HIF-1α nuclear accumulation and transactivation [77]. It is known that VEGF is transcriptionally up-regulated by HIF-1α. Overexpression of filamin A increased recruitment of HIF-1α to VEGF promoter and augmented angiogenesis in a tumor xenograft model [77]. Another crosstalk involves vasohibin-1 (VASH1), an angiogenesis inhibitor generated by VEGF-stimulated endothelial cells [78,79]. It was reported that hypoxia inhibits VEGF-induced VASH1 expression [79]. Interestingly, tumor cells inactivate VASH1 through calpain-dependent cleavage of VASH1 in EC-tumor cell co-culture experiments [78]. These calpain central tumor cell-EC crosstalks to facilitate angiogenesis are summarized in Fig. 2.

To study the involvement of endothelial calpains in tumor angiogenesis, Miyazaki et al. collected tumors and nearby normal tissues from patients with malignant astrocytoma, colon and lung adenocarcinomas. Immunostaining of calpastatin illustrated that the expression level of calpastatin was significantly reduced in endothelial cells of tumor vessels compared to nearby normal vessels [44]. They further generated transgenic mice that harbor endothelial cell specific transgene of calpastatin. In these animals, tumor angiogenesis was attenuated in a Lewis lung carcinoma allograft transplantation model. It turns out that calpastatin inhibits VEGF-C production through calpain/ SOCS3/STAT3 [44]. These results provide more evidences that regulation of calpain pathway is important in tumor angiogenesis (summarized in Fig. 2).

4.3.2. Calpains in diabetic wound healing

Diabetic food ulcer, one of the most common complications of diabetes mellitus, affects 15% of people with diabetes [80]. It is also the leading cause of amputations among diabetic patients [81,82]. One of the major causes of diabetic foot ulcer is impaired wound healing, which is characterized by impaired growth factor production and defective angiogenesis [82–85]. It is known that the expression of growth factors and their receptors (such as VEGF, platelet-derived growth factor (PDGF)/PDGF receptor, FGF/FGF receptor, EGF) is up-regulated in the wounded area during physiological repair [83]. However, the up-regulation of growth factors and their receptors is absent in diabetic wounds. The synthesis of PDGF and FGF like growth factors was down-regulated in STZ-induced diabetic wound [83,86]. The abundance of VEGF was decreased in wounded area throughout wound healing process in the db/db mice [87]. Galkowska et al. have compared the expression of growth factors and their receptors in the margin skin tissue of diabetic foot ulcers with normal non-diabetic foot skin by immunohistochemistry [84]. They reported down-regulation of PDGF receptor and TGF-pl. There was a similar trend for VEGF/ VEGFR2, EGF and FGF [84]. In accordance with the impaired production of growth factors, angiogenesis process is also delayed in diabetic wound [88–90]. To promote wound healing through angiogenesis, multiple strategies have been tested. Administration of growth factors has been shown to be effective [45,91]. Others and we have shown that VEGF activates PI3K/AMPK/Akt/eNOS cascade to induce NO production, which in turn mediates angiogenesis and wound healing [14,92,93]. Topical application of VEGF accelerated skin wound healing in both type 1 and type 2 diabetic models [45,80]. This strategy and other approaches discussed below to improve diabetic wound healing are summarized in Fig. 3.

To further accelerate wound healing, an alternative approach is to facilitate angiogenesis by targeting downstream pathways of growth factors. We have recently shown that VEGF signaling is regulated by a calpain/PTPlB/VEGFR2 feedback mechanism, which can be employed to enhance VEGF signaling to facilitate therapeutic angiogenesis [45]. PTP1B activity is up-regulated in diabetic wound to constrain VEGFR2, while application of PTP1B inhibitor accelerated VEGF-dependent diabetic wound healing [45]. To test the effect of calpain in wound healing, we directly applied plasmids that encode human calpain cDNA to the wound bed. As expected, we found that calpain overexpression in wound bed accelerated VEGF-induced wound healing in STZ-induced diabetic mice [45]. Data from calpastatin transgenic mice confirmed the importance of calpain in angiogenesis and wound healing.

Global calpastatin transgenic mice showed impaired angiogenesis and a striking delay in wound healing [70]. Moreover, calpain 2 is indispensable to lymphangiogenesis [58], enhancement of which contributed to accelerated diabetic wound healing [80,94,95].

Studies examining roles and mechanisms of calpains in pathophysiological angiogenesis, including models and approaches employed, are summarized in Table 1.

5. Other mechanisms that regulate calpain activity in endothelial cells

5.1. Oxidized LDL, phospholipids and mechanical stress

In addition to growth factors and hypoxia, calpains can be activated in endothelial cells by oxidized low-density lipoprotein (oxLDL), phospholipids or mechanical stress. OxLDL induces calpain activation in endothelial cells through elevated calcium concentration [96,97]. In human atherosclerotic plaques, calpain activity was identified in apoptotic cells [98]. In addition, oxLDL-induced endothelial cell apoptosis can be partially blocked by PD151746, a calpain 1 inhibitor [99]. These results suggest that at least calpain 1 activation is involved in ox-LDL-induced endothelial apoptosis, which has long been considered as an important regulator of the initiation and progression of atherosclerotic lesions. On the other hand, calpain 2 has been reported to regulate endothelial adherence junctions (through cleavage of VE-cadherin) and promote atherogenesis. Recently, Miyazaki et al. have shown that endothelial calpain 2 up-regulation is associated with more severe atherosclerotic lesions in patients [100]. In LDL receptor (LDLR) deficient mice, high cholesterol fed animals showed elevated expression of endothelial calpain 2. Administration of calpain inhibitors (calpeptin, ALLM) on the other hand significantly limited lesion formation in high cholesterol diet-treated LDLR knockout or Apolipoprotein E deficient mice [100]. Similarly, lysophosphatidylcholine, the major lipid constituent of oxLDL, can activate calpain 2 and induce cleavage of VE-cadherin [100,101]. Taken together, activation of calpain 1 and calpain 2 by oxLDL contributes to atherogenesis. Though a causal role of angiogenesis in atherosclerosis has not been established, there is strong evidence that the development of plaques is associated with neovascularization within the plaque [102–105].

It has also been reported that calpain 2 is activated by physiological shear stress through elevated intracellular calcium [106,107]. Calpeptin impaired shear stress-induced focal adhesion polarization and cell alignment under shear conditions [107]. Shear stress, combined with sphingosine 1-phosphate, induces calpain membrane translocation and MTP-MMP1 activation in endothelial cells [52]. On the other hand, Mayazaki et al. have demonstrated that calpain 2 antagonizes RhoA overactivation and endothelial barrier dysfunction in response to disturbed flow [108]. Overall calpain 2 activation under different flow patterns seem to exert protective signaling via differential mechanisms.

5.2. Ischemic retinopathy

Calpains have been reported to contribute to hypoxia-derived retina cell death in ischemic retinopathy [109,110]. Using an oxygen-induced retinopathy (OIR) model, Hoang et al. demonstrated that ischemic hypoxia activates calpain in retinal endothelial cells and disrupts actin cytoskeleton in human retinal microvascular endothelial cells [46]. Moderate inhibition

(30–35%) of calpain activity results in formation of functional neovasculature. Further investigation revealed that calpain inhibitor MDL28170 and calpastatin peptide improved organization and alignment of actin cytoskeleton both in vitro and in vivo [46]. The role of calpains in ischemic retinopathy was also reported by another independent group using the OIR model [44]. Endothelial specific transgene of calpastatin abolished OIR-induced vascular tufts through down-regulation of calpain 1-dependent cleavage of SOCS3, followed by inhibition of STAT3 and VEGF-C expression [44]. These results suggest that targeting calpain system (either by application of calpain inhibitor or overexpression of calpastatin) is beneficial for the normalization of angiogenesis in ischemic retinopathy.

6. Differential strategies targeting endothelial calpain and calpain-dependent pathways for different pathophysiological conditions

Calpain 1 and calpain 2 are ubiquitously expressed in human tissues. For therapeutic purposes related to angiogenesis, endothelial targeted strategies of modulating calpain activity may be beneficial. Restrain of calpain activity inhibits tumor growth and atherogenesis, and promotes formation of normal vasculature in ischemic retinopathy. On the other hand, activation of calpain is aimed to treat diabetic wound healing and protect endothelial function under disturbed flow. Therefore, differential strategies of regulating endothelial calpain/ angiogenesis are necessary for therapeutic control of different pathophysiological conditions.

One of the options enabling endothelial cell-targeted delivery involves generating genetically modified vectors. Plasmids or viral vectors-based gene targeting could specifically modulate gene expression in certain type of cells, with the help of tissue-specific promoters/ enhancers, and sequences with physiological sensing elements (such as those responsive to hypoxia, shear stress) [111,112]. Localized intraspinal injection into rats of lentiviral vector encoding calpain 1 resulted in sustained ability of proteolysis (activated NF-κBp65 after IκB being cleaved by calpain 1) up to 7 weeks after injection [113,114]. It has been reported that hypoxia response element from the promoters of multiple genes (such as erythropoietin, phosphoglycerate kinase-1, and VEGF) has been used as a hypoxia sensitive enhancer to promote transcription of the delivered gene of interest [111,115–117]. Very recently, lentiviruses that are pseudotyped with endothelial-specific envelopes recognizing endothelial cell surface marker CD105 have been shown to be efficient and specific in endothelial delivery after systemic injection [118]. Tumor endothelial cells were specifically targeted upon intratumoral injection in mice carrying a vascularized human tumor xenograft [118]. Most recently, the emergence of CRISPR/Cas9 gene editing system further increases the possibility of in vivo genome editing [119–121]. Yin et al. recently reported that systemic delivery of nanoparticle-conjugated Cas9 mRNA and sgRNA/HDR sequences by AAV provided efficient genome editing and less off-target editing in diseased mice [122].

Especially, tumor cells are known to have lower pH (6.2–6.9) than normal tissues (7.3–7.4) due to the glycolysis under anaerobic environment [123]. Modified nanoparticles can be used as the vehicle of delivery which is capable of pH-dependent drug release and has been shown to effectively inhibit xenografted tumor growth [124–126]. Another approach to achieve local regulation is to apply treatment in situ. In a diabetic wound healing model, topical administration of recombinant VEGF and calpain plasmids accelerated wound closure [45]. This local application is transient and restrained only to wounded area. This approach is especially applicable to dermal treatment.

In conclusion, calpain 1 and calpain 2 play important roles in VEGF-induced angiogenesis in endothelial cells. Blockade of calpain 2 impairs, while activation of calpain 2 promotes, VEGF-induced angiogenesis. In animal models, inhibition of calpain 1 and calpain 2 by calpastatin transgene specifically expressed in endothelial cells efficiently attenuated tumor angiogenesis and tumor growth [44]. Abrogation of calpain activity reduced progression of atherosclerosis and ischemic retinopathy [46,100,127]. On the other hand, activation of calpain pathway accelerated diabetic wound healing, and is dispensable to protect from disturb flow-induced endothelial cell barrier dysfunction [45,108]. Therefore, disease-specific modulations of calpain, combined with endothelial cell-targeted delivery techniques, may prove to be promising strategies for the treatments of pathophysiological conditions associated with angiogenesis.

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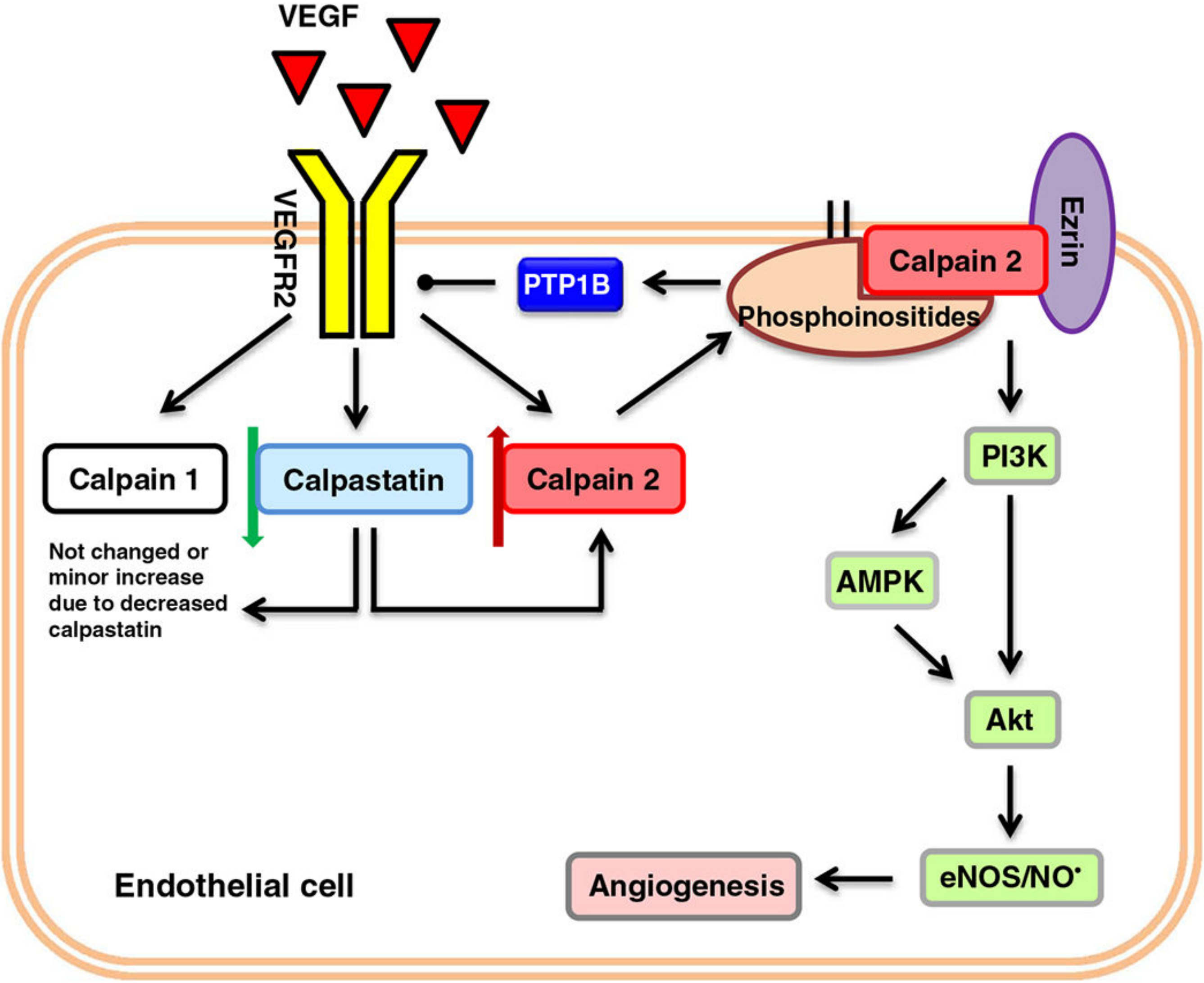

Fig. 1.

VEGF activates angiogenesis through calpain 2. VEGF/VEGFR2 activates calpain 2, not calpain 1, in endothelial cells. Calpain 2 activation Involves binding to Ezrln and phosphoinositides on the cell membrane. VEGFR2 Is negatively regulated by a calpain 2/PTP1B feedback loop. Calpain 2 mediates VEGF-lnduced activation of PI3K/AMPK/Akt/eNOS pathway and consequent nitric oxide (NO) production and angiogenesis.

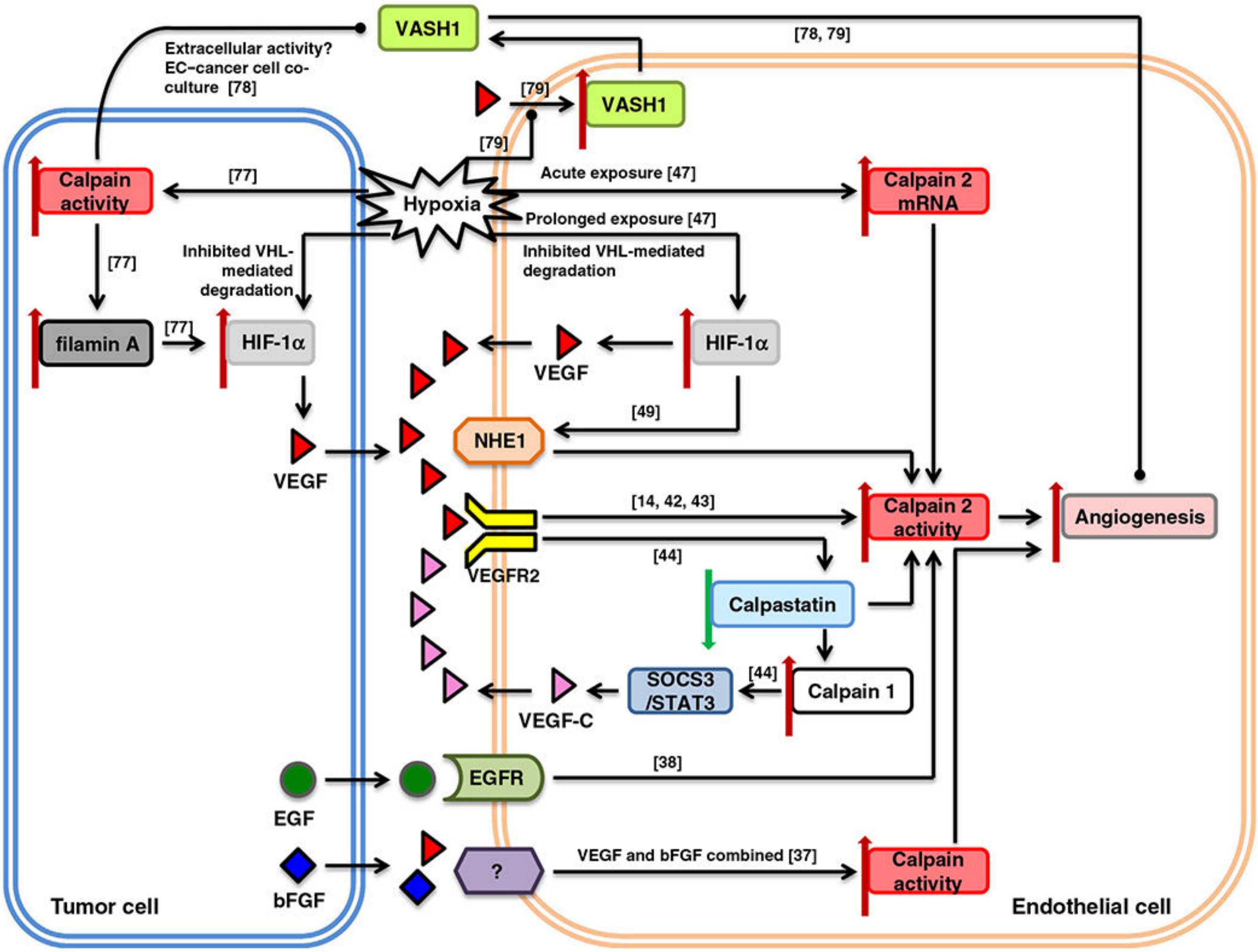

Fig. 2.

Calpain central signaling pathways in tumor angiogenesis. Under hypoxic conditions, von Hippel-Lindau (VHL)-mediated HIF-1α degradation is inhibited, leading to accumulation of HIF-1α in both tumor cells and endothelial cells. VEGF expression is transcriptionally up-regulated by HIF-1α. Hypoxia also increases calpain activity in tumor cells. The cleavage of filamin A, a substrate of calpain. facilitates HIF-1α nuclear translocation and enhances HIF-1α transactivation, resulting in up-regulation of VEGF expression and secretion. On the other hand, hypoxia induces calpain 2 activation in endothelial cells. During acute exposure, hypoxia directly upregulates calpain 2 mRNA, while prolonged exposure to hypoxia increases calpain 2 activity through HIF-la-induced VEGF and NHE1 expression. Other growth factors secreted by tumor cells, such as EGF and bFGF, are able to activate endothelial calpain 2 as well. Down-regulation of calpastatin causes calpain 1-dependent SOCS3 cleavage and VEGF-C production through STAT3 phosphorylation. Activation of endothelial calpain 2 leads to enhanced tumor angiogenesis. Another calpain-dependent up-regulation of angiogenesis involves VASH1. Hypoxia inhibits VEGF-induced expression of VASH1 in endothelial cells. Secreted VASH1 undergoes cleavage by tumor calpain. which impairs its anti-angiogenic function.

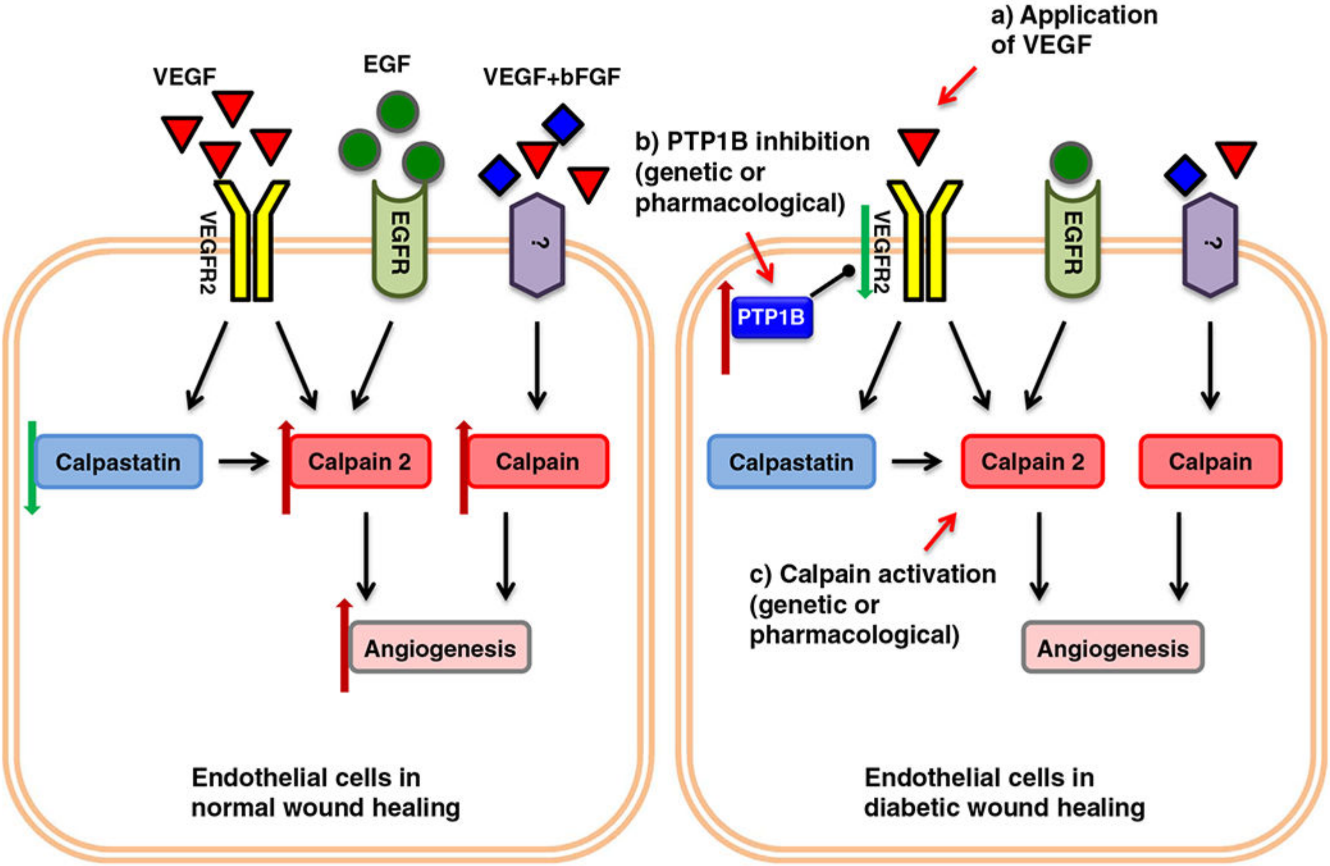

Fig. 3.

Mechanisms of impaired angiogenesis in diabetic wound healing. Normal wound healing can be facilitated by VEGF via VEGFR2/calpain 2/eNOS/angiogenesis axis (details see Fig. 1). In diabetes, impaired wound healing is characterized by reduced growth factors expression (VEGF, FGF, and EGF), and increased PTP1B activity to inhibit VEGFR2. To promote angiogenesis in diabetic wound healing, the following strategies can be employed; a) supplementation of VEGF; b) genetic or pharmacological approaches to inhibit PTP1B to increase VEGFR2-dependent angiogenic signaling; or c) genetic or pharmacological approaches to activate calpain.

Table 1

Roles and mechanisms of calpains in pathophysiological angiogenesis.

Pathophysiologic al conditions	In vitro model	In vivo model	Way of intervention	Experimental results	Signaling pathways	References
Wound healing	BAECs.	STZ-induced diabetic mice.	In vitro and in vivo: application of calpain inhibitor ALLN.	In vitro and in vivo: delayed VEGF-induced wound closure.	VEGF/calpain/angiogenesis/wound healing	[45]
	Monolayer wound scratch assay.	Skin wound healing assay.	In vivo: over expression of calpain 1 and calpain 2.	In vivo: accelerated VEGF-induced wound closure.		
Wound healing	N/A	Global calpastatin transgenic mice; Skin wound healing assay.	In vivo: calpastatin transgene.	In vivo: calpastatin Tg mice have impaired cell proliferation, and decreased blood vessel density. Calpastatin Tg mice showed delayed angiogenesis and wound healing.	Calpastatin/calpain/angiogenesis/wound healing	[70]
Wound healing	Human microvascular cell line (HMEC-1);	N/A	In vitro: IP-10 treatment.	In vitro: leads to β3 integrin cleavage and tube dissociation.	IP-10/CXCR3/calpain 1/(β3 integrin/tube dissociation (non-VEGF).	[72]
	Tube formation.		In vitro: application of CI-1 (a pan-calpain inhibitor), CI-IV (calpain 2 inhibitor), BAPTA/AM.	In vitro: IP-10-induced tube dissociation was prevented by BAPTA/AM and CI-1, but not CI-IV.		
				In vitro: IP-10-induced β3 integrin cleavage was blocked by BAPTA/AM and CI-1.		
Tumor angiogenesis	Melanoma cells.	Tumor xenograft model for angiogenesis.	In vitro and in vivo: over expression of filamin A (calpain substrate).	In vitro: overexpression of filamin A increased HIF-1α transactivation and VEGF expression (mRNA and secretion) in hypoxia.	Calpain/filamin A/HIF-1α activity/VEGF/angiogenesis	77
	Exposure to hypoxia.					
					In vivo: increased HIF-1α activity (expected increased VEGF production) and angiogenesis in a tumor xenograft model derived from filamin A overexpression.	
			In vitro: application of calpain inhibitor calpeptin.	In vitro: calpeptin inhibited filamin A cleavage, and HIF-lα nuclear accumulation and transactivation in melanoma cells (VEGF is transcriptionally regulated by HIF-lα).		
Tumor angiogenesis	Human aortic endothelial cells.	Endothelial-specific calpastatin Tg mice.	In vitro: calpastatin siRNA transfection and IL-6 treatment.	In vitro: calpastatin siRNA facilitated calpain 1-induced cleavage of SOCS3, leading to VEGF-C production through amplified IL-6-driven STAT3 signals.	Calpastatin/calpain 1/SOCS3/STAT3 (induced by IL-6)/VEGF-C/ angiogenesis	[44]
	Tube formation, cell mobility, proliferation.	Lewis lung carcinoma xenograft model.		Calpastatin siRNA also improved angiogenic responses (tube formation, cell mobility, proliferation) in the presence of IL-6.		
			In vivo: calpastatin transgene.	In vivo: calpastatin Tg declined phosphorylation of STAT3 in tumors, up-regulated SOCS3 protein expression, and decreased VEGF-C production in the tumor neovessels. Calpastatin Tg suppressed tumor angiogenesis.		
Atherosclerosis	HMEC-1	N/A	In vitro: treatments of oxLDL and calpain inhibitor calpeptin.	In vitro: calpeptin inhibited oxLDL-induced calpain activation, Bid cleavage, cytochrome C release, caspase-3 activation, and cytotoxicity.	OxLDL/calcium/calpain/Bid/cytochrome C/caspase-3/apoptosis	[97]
Atherosclerosis	HMEC-1	N/A	In vitro: treatments of oxLDL and calpain inhibitor PD 151746. (PD 151746 has more than 20-fold selectivity for calpain 1 over calpain 2.)	In vitro: PD 151746 decreased oxLDL-induced cytotoxicity. OxLDL-induced Bid cleavage was prevented by PD 151746.	OxLDL/calpain/Bid/apoptosis	[99]
Atherosclerosis	HUVEC	LDLR-KO or apoE-KO mice, fed with high cholesterol food (HCD).	In vitro: transfection of calpain 2 siRNA and treatments of LPC and calpeptin.	In vitro: LPC-induced VE-cadherin cleavage and hyperpermeability can be blocked by calpeptin or calpain 2 siRNA. LPC-induced dissociation of beta-catenin/VE-cadherin was attenuated by calpeptin. Calpain 2 directly cleaves VE-cadherin.	HCD or LPC/calpain 2/VE-cadher in/beta-catenin/endothelial adherence junctions/atherosclerosis	[100]
			In vivo: administration of calpain inhibitors (ALLM and calpeptin).	In vivo: long-term administration of calpain inhibitors attenuated VE-cadherin disorganization and atherosclerotic lesion development.		
			In vivo transfection of calpain 2 siRNA.	In vivo: prevented disorganization of VE-cadherin and proatherogenic hyperpermeability in aortic endothelial cells.		

Pathophysiologic al conditions	In vitro model	In vivo model	Way of intervention	Experimental results	Signaling pathways	References
Ischemic retinopathy	Human dermal microvascular endothelial cells (HDMEC).	Endothelial-specific calpastatin Tg mice.	In vitro: calpastatin siRNA. IL-6 treatment.	In vitro: calpastatin siRNA increased IL-6-induced tube formation.	calpastatin/calpain 1/SOCS3/STAT3/VEGF-C/angiogenesis	[44]
	Tube formation.	Oxygen-induced retinopathy (OIR) model.	In vivo: endothelial-specific calpastatin transgene.	In vivo: calpastatin Tg abolished OIR-induced pathological angiogenesis (vascular tufts formation), up-regulated SOCS3 expression and STAT3 phosphorylation, and diminished VEGF-C expression (mRNA).		
Ischemic retinopathy	Human retinal microvascular endothelial cells (RMVEC).	Oxygen-induced retinopathy model.	In vitro: application of calpain inhibitors: MDL28170, calpastatin.	In vitro: hypoxia induces calpain activation in RMVECs, along with disrupted actin cytoskeleton. Modest inhibition of calpain by MDL28170 or calpastatin improved actin cytoskeletal organization and capillary morphogenesis in a 3D collagen matrix assay.	Calpain/cell cytoskeleton (organization and alignment of actin cables)/ angiogenesis	[46]
	Capillary-like tube growth in 3D collagen matrix.					
	Exposure to hypoxia.		In vivo: application of MDL28170, PD150606, ALLN.	In vivo: moderate calpain inhibition (by MDL28170, PD150606 or ALLN) improved organization and alignment of actin cytoskeleton and promoted formation of functional neovasculature.		