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Review

Dysregulation of Calpain Proteolytic Systems Underlies Degenerative Vascular Disorders

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Chronic vascular diseases such as atherosclerosis, aneurysms, diabetic angiopathy/retinopathy as well as fibrotic and proliferative vascular diseases are generally complicated by the progression of degenerative insults, which are characterized by endothelial dysfunction, apoptotic/necrotic cell death in vascular/immune cells, remodeling of extracellular matrix or breakdown of elastic lamella. Increasing evidence suggests that dysfunctional calpain proteolytic systems and defective calpain protein metabolism in blood vessels contribute to degenerative disorders. In vascular endothelial cells, the overactivation of conventional calpains consisting of calpain-1 and -2 isozymes can lead to the disorganization of cell-cell junctions, dysfunction of nitric oxide synthase, sensitization of Janus kinase/signal transducer and activator of transcription cascades and depletion of prostaglandin I_2, which contributes to degenerative disorders. In addition to endothelial cell dysfunctions, calpain overactivation results in inflammatory insults in macrophages and excessive fibrogenic/proliferative signaling in vascular smooth muscle cells. Moreover, calpain-6, a non-proteolytic unconventional calpain, is involved in the conversion of macrophages to a pro-atherogenic phenotype, leading to the pinocytotic deposition of low-density lipoprotein cholesterol in the cells. Here, we discuss the recent progress that has been made in our understanding of how calpain contributes to degenerative vascular disorders.

Key words: Calpastatin, Vascular inflammation, Pathological angiogenesis, Extracellular matrix, Oxidative stress

Introduction

Majority chronic vascular diseases, including atherosclerosis, aneurysms, diabetic angiopathy and proliferative retinopathy as well as fibrotic vascular diseases, harbor unique and common degenerative insults[1-6]. For example, atherosclerotic vessels frequently accompany dysfunction of the vascular endothelial cells (ECs), accumulation of cholesterol-enriched cellular apoptotic bodies, remodeling of the extracellular matrix (ECM) and breakdown of elastic lamella[2]. Although the deposition of cholesterol is unique to atherosclerotic diseases, the endothelial dysfunction and ECM remodeling are frequently detected in other vascular diseases, such as diabetic angiopathy and vascular fibrosis. In general, vascular degenerations are driven by chronic inflammatory responses and accompanying reactive oxygen species (ROS)-mediated oxidative stress[7]; thus, many researchers have tested anti-oxidative agents in clinical trials. Unfortunately, these anti-oxidative trials have thoroughly failed to reverse chronic vascular diseases[8], although they were effective in acute inflammation, such as the acute phase of stroke[9]. Therefore, it is necessary to investigate how blood vessels shift toward degenerative status in chronic disease. In this regard, growing evidence suggests that the calpain proteolytic systems, comprised of Ca^{2+}-dependent intracellular proteases and a calpain endogenous inhibitor, critically contribute to degenerative vascular disorders. Calpains can be potentiated in the presence of pathophysiological stressors, such as inflammatory cytokines, growth factors, bioactive lipids and hypoxic stimulus[10, 11]. As a result, dysregulation of these systems might be responsible for the vascular disorders noted above. In this review article, we discuss the current achievements in understanding the pathophysiology of degenerative vascular disorders, with a particular focus on the calpain proteolytic system.

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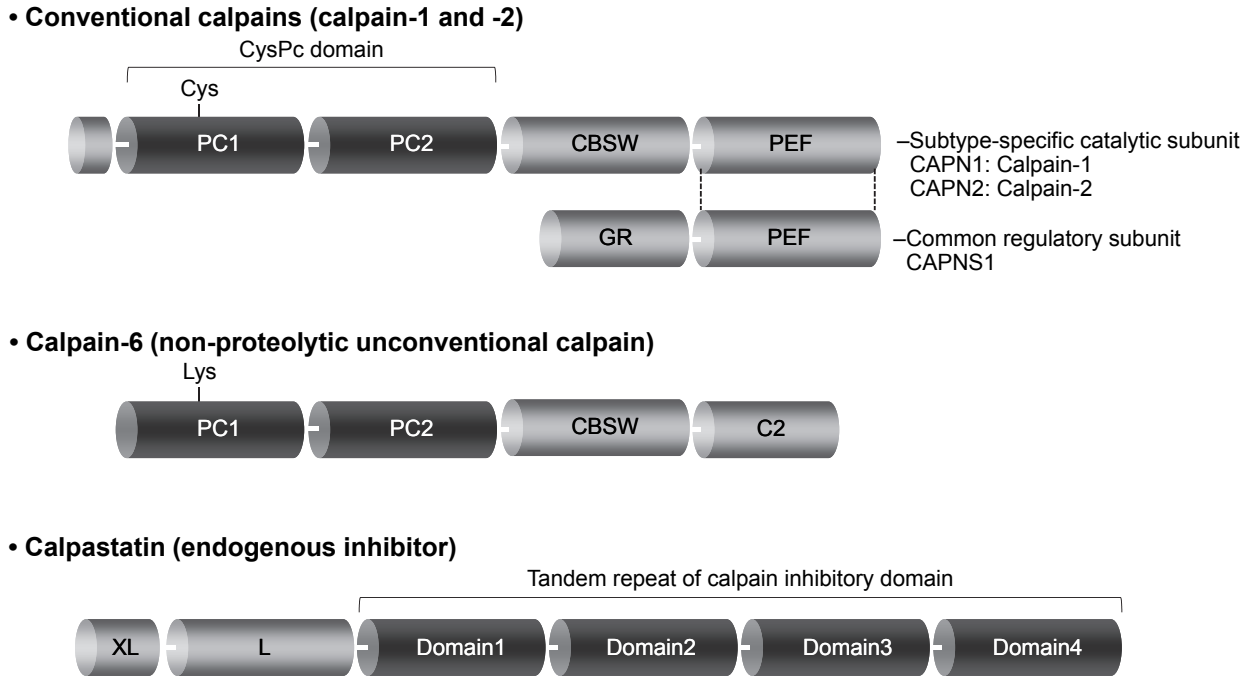

Fig. 1. Vascular calpain systems. Conventional calpains are localized to the majority of the vascular component cells, including vascular endothelial cells (ECs), vascular smooth muscle cells (VSMCs) and fibroblasts. Calpain-6, a non-proteolytic unconventional calpain, is inducible in foamy macrophages in athero-prone vessels. Calpastatin, an endogenous inhibitor, colocalizes with conventional isozymes, and downregulates their proteolytic activity. PC: protease core domain, CBSW: calpain-type β-sandwich domain, PEF: penta-EF-hand domain, GR: glycine-rich domain, C2: C2 domain, XL: XL domain, L: L domain.

Vascular Calpain Systems

Calpains comprised of 15 homologues in mammals can be categorized as conventional and unconventional isozymes. Conventional calpains consist of two ubiquitous isozymes, calpain-1 and -2, which require micromolar and millimolar levels of Ca^{2+} for half-maximal activation, respectively[12-14]. These ubiquitous isozymes are comprised of heterodimers containing a common regulatory subunit, *CAPNS1*, and subtype-specific large subunits, *CAPN1* and *CAPN2*, which function as the catalytic subunits of calpain-1 and -2 isozymes, respectively[12-14] (**Fig. 1**). Conventional calpains are localized to the majority of vascular systems, including veins[15], arteries[16] and capillaries[17], and are also expressed in ECs[16, 18], vascular smooth muscle cells (VSMCs)[19] and adventitial fibroblasts[20]. In addition to the vascular component cells, calpains are localized to immune cells[21, 22], which can be recruited into blood vessels in response to inflammatory insults. In addition to the conventional calpain isozymes, calpastatin, an endogenous calpain inhibitor, colocalizes with the proteases, and negatively regulates their proteolytic activity[12-14]. It was reported that deficiency of the *Capn1* gene in mice resulted in normal growth with a reduction in platelet aggregation and clot retraction[23]; therefore, calpain-1 does not play a vital role in these functions. In contrast, *Capn2*-deficient mice showed embryonic lethality between the morula and blastocyst stages[24]. Similar to *Capn2*, deficiency of *Capns1* was embryonic lethal and accompanied by disorders in cardiovascular development[25]. Takano *et al.* subsequently reported that conditional knockout mice, in which *Capn2* was expressed in the placenta but not in the fetus, survived to adulthood[26]. Thus, calpain-2 is unlikely to play an essential role in cardiovascular development in the fetus.

In general, physiological substrates of conventional calpains are hard to predict, because the proteases do not strictly recognize unique consensus amino acid sequences or structural motifs in their substrates. Earlier enzymatic investigations reported that conventional calpains post-translationally proteolyze a variety of substrates, including the focal adhesion proteins talin[27] and vinculin[28], leading to alterations in cell motility and morphology. Furthermore, the calpain-dependent post-translational regulation of signaling molecules such as focal adhesion kinases[29], protein kinase C[30] and inhibitor of κB (IκB)[31, 32] have been described previously. It was documented that conven-

tional calpains proteolytically degrade the EC-derived angiostatic peptide vasohibin-1 in tumor cells[33]. Recently, we identified VE-cadherin, an inter-endothelial adhesion molecule[34], and suppressor of cytokine signaling 3 (SOCS3), an endogenous inhibitor of Janus kinase (JAK)/signal transducer and activator of transcription (STAT) cytokine signaling[35] as substrates of the calpain system. These data suggest that conventional calpains recognize diverse substrates and modify multiple signaling cascades in cells, and it is suspected that their impact on cellular functions is dependent upon cell type or environmental factors, such as the type of inducer. Please refer to the following excellent reviews for extensive reviews of the molecular basis of conventional calpains[12-14].

In addition to conventional isozymes, 13 species of calpain catalytic subunit have been identified in mammals. These unconventional isoforms are further categorized into ubiquitous and tissue-specific forms according to their tissue localization in vertebrates[12-14]. Compared with the conventional isoforms, the roles of the unconventional isoforms in vascular regulation have been less extensively investigated, probably because of their low expression in the vasculature. Indeed, our preliminary data showed that the expression levels of unconventional calpains in murine normal healthy aorta are much lower in comparison with those of *Capn1*, *Capn2*, *Capns1* and *Cast* (our unpublished observations); nevertheless, it is suspected that certain unconventional isozymes can be potentiated under pathophysiological conditions. For instance, calpain-6 is induced in foamy macrophages in response to inflammatory stimuli during atherogenesis[36], whereas this molecule was reportedly localized to fetal skeletal muscle, cartilage and heart[37] as well as the placenta in adults[38] but not to blood vessels during normal physiological status. This subtype appears to lack proteolytic activity, as the cysteine residue in its active core is substituted with lysine[12-14] (**Fig. 1**). Tonami *et al.* identified that the deletion of *Capn6* in mice promoted the development of embryonic skeletal muscle[37]. Furthermore, calpain-6 was induced during the regeneration of skeletal muscles in adult mice after cardiotoxin-induced degeneration, and *Capn6* deficiency accelerated skeletal-muscle regeneration in mice[37]; however, the role of calpain-6 in the physiological regulation of vascular systems is currently poorly understood.

Calpain-10 is ubiquitously expressed and is associated with apoptosis in pancreatic islet cells[39], mitochondrial dysfunction[40], insulin secretion from pancreatic islets[41, 42], and the oxidative utilization of glucose in skeletal muscle[43]. *Capn10* deficiency in the SM/J mouse strain ameliorated insulin resistance and reduced blood glucose levels[44]. Furthermore, a genome-wide analysis has shown that a *CAPN10* polymorphism is involved in insulin resistance, dyslipidemia, and high free fatty acid levels in a Japanese population[45]; obesity in a Scandinavian population[46]; and free fatty acid levels in a Finnish population[47]. It is likely that genetic variation in the *CAPN10* locus confers higher cardiovascular disease risk to type 2 diabetes mellitus patients[48]. Therefore, calpain-10 might induce diabetic angiopathy through its diabetogenic actions, whereas the direct role of this molecule in vascular regulation is unknown.

Contribution of Calpain Proteolytic Systems to Degenerative Vascular Disorders

Atherosclerosis and Aneurysmal Diseases

Atherosclerosis is a vascular disease characterized by the intimal thickening of systemic arteries, including the coronary and cerebral arteries as well as the aorta[49, 50]. Vulnerable and occlusive atherosclerotic plaques can lead to lethal cardiovascular events, including myocardial infarction and stroke, two primary causes of morbidity and mortality worldwide. Because many pathogenic cues contribute to atherogenesis[50], it is difficult to precisely define the cause of atherosclerosis; however, the majority of investigations are based on the hypothesis that ROS-mediated oxidative stress in atherosclerotic lesions induces various inflammatory elements, such as endothelial adhesion molecules (e.g. intercellular adhesion molecule-1, vascular cell adhesion molecule-1, E-selectin), inflammatory cytokines and chemokines, through redox-sensitive transcription factors[50]. The overexpression of adhesion molecules in ECs facilitates leukocyte adhesion to ECs and monocyte chemoattractant protein-1 potentiates cellular motility in monocytes/macrophages, thereby accelerating the infiltration of these cells into lesions. Recruited macrophages incorporate low-density lipoprotein (LDL) cholesterol through micro/macro pinocytosis and phagocytosis pathways as well as scavenger receptor-mediated endocytosis[51]. Internalized endocytic vesicles fuse with lysosomes; accordingly, the enclosed lipoproteins are degraded through lysosomal digestion. After lysosomal degradation, acyl-coenzyme A: cholesterol acyltransferase 1 converts free cholesterol to highly hydrophobic cholesterol esters, thereby forming lipid-enriched foam cells[52]. In addition to the cholesterol deposition in macrophages, immune responses in immunocompetent cells including T cells, B cells and mast cells in the lesions orchestrate atherogenicity[53]. For instance, type 1 and type 2 T-helper cells polarize macrophages toward M1 and M2 subsets, respectively[54]. It was reported that M1 macrophages largely contribute to the progression of atherogenesis through its pro-inflam-

matory ability. Furthermore, inflammasomal production of interleukin (IL)-1β reportedly facilitates development of atherosclerotic lesions[55]; thus, innate immunity as well as adaptive immunity can modify the atherosclerotic vascular inflammation. We have previously reported that *Capn2*, *Capn6* and *Capn9*, but not other family members, are induced in the pro-atherogenic aortae of high cholesterol diet-fed *Ldlr*$^{-/-}$ mice[36]. Among these calpain species, conventional calpain-2 is induced in response to oxidized or enzymatically-modified LDL or its component lysophosphatidylcholine, and is abundant in ECs in mouse and human atheromas[34]. The calpain inhibitors calpeptin or ALLM (N-acetyl-L-leucyl-L-leucyl-L-methioninal) appear to prevent pro-atherogenic barrier dysfunctions in ECs, and reduce the recruitment of circulating monocytes into atherosclerotic lesions; thus, the administration of these inhibitors to high cholesterol diet-fed *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice suppresses the development of atherosclerotic lesions. In these cases, barrier dysfunction is caused by the calpain-2-induced proteolysis of VE-cadherin, because VE-cadherin promotes homophilic adhesion between ECs, thereby forming an interendothelial barrier[56]. Subramanian *et al*. also noted that atherosclerotic lesion progression in angiotensin Ⅱ-infused *Ldlr*$^{-/-}$ mice was impaired by the calpain inhibitor BDA-410[57]. Transgenic overexpression of calpastatin significantly attenuated angiotensin Ⅱ- or hypercholesterolemia-induced atherosclerotic development in *Ldlr*$^{-/-}$ mice[58]. Furthermore, myeloid calpain-1 or leukocyte calpain-2 were involved in angiotensin Ⅱ-induced atherosclerosis[58]. In this case, calpain inhibition reduced the motility, mitosis and nuclear factor-κB (NF-κB)-dependent inflammatory responses in isolated macrophages. Collectively, it is likely that conventional calpains are involved in the pro-atherogenic regulation of EC and macrophages.

In addition to the pro-atherogenic actions of the conventional calpains, we recently identified the contribution of unconventional calpains to the pathogenesis of atherosclerosis[36]. Indeed, *Capn6* ablation prevented the development of atherosclerotic lesions in mice, whereas *Capn9* knockout did not affect the phenotype. Calpain-6 is localized to macrophages, but not to other vascular component cells in advanced human and murine atheromas. Consistently, bone marrow transplantation experiments have demonstrated that myeloid *Capn6* is responsible for atherogenesis. Calpain-6 in macrophages is inducible in response to inflammatory cytokines such as tumor necrosis factor-α (TNF-α), and directly binds to CWC22, an essential loading factor for the exon-junction complex (EJC). CWC22 escorts the EJC to the nucleus, which is necessary for spliceosome-driven mRNA splicing. The efficiency of mRNA splicing was reduced after silencing CWC22[59]; thus, CWC22 is indispensable for mRNA splicing. The binding of calpain-6 with CWC22 in the cytoplasm prevented the nuclear translocation of CWC22. Accordingly, the splicing efficiency of their target genes, including Rac1, was decreased in the presence of calpain-6, which conferred atherogenicity to foamy macrophages. Rac1 is a negative regulator of the pinocytosis pathway; thus, the impaired nuclear translocation of CWC22 by calpain-6 augments the pinocytotic uptake of native LDL in macrophages. Importantly, the ablation of calpain-6 substantially decreased pinocytotic activity in macrophages in murine atherosclerotic lesions[36]. Immunohistochemistry of human aortic atheromas showed that the nuclear localization of CWC22 was diminished in macrophages in advanced atheromas but not in cells of mild atherosclerotic lesions, while calpain-6 was upregulated in macrophages in advanced atheromas[36]. Thus, the calpain-6-induced disruption of CWC22/EJC in macrophages translates to human atherosclerotic lesions. A previous review articles have further details of calpain-mediated regulation of atherosclerosis[10] and pro-atherogenic cholesterol handling in macrophages[60].

Abdominal aortic aneurysm, a representative atherosclerotic disease, is a major cause of cardiovascular death and the tenth leading cause of death in men over 65 years of age in western countries[61]. Excessive oxidative stress in atheroprone arteries facilitates the accumulation of matrix metalloproteinases (MMPs) and subsequent remodeling of vascular tissues, which is sometimes a threshold process in aneurysmal diseases[62, 63]. Indeed, macrophage- and vascular cell-derived MMPs-9 and -12 are considered major exacerbators of aneurysmal diseases[64, 65], because they break down vascular structures, including the elastic lamellae and basement membrane, which leads to vascular dissection[62, 63]. Previous proteomic analyses demonstrated the contribution of calpain-2 to aortic aneurysms in Marfan syndrome[66]; thus, it was suspected that conventional calpains cause atherosclerotic aortic aneurysms. Indeed, the pharmacological intervention of conventional calpains by BDA-410 ameliorated aortic dissection in angiotensin Ⅱ-infused *Ldlr*$^{-/-}$ mice, concomitant with the reduction of MMP12, IL-6 and MCP-1 levels in the vessels[67]. In contrast, the myeloid-specific transduction of calpastatin or deficiency of calpain-1 as well as the macrophage-specific deficiency of calpain-2 had no effect on aneurysmal formation in angiotensin Ⅱ-induced *Ldlr*$^{-/-}$ mice, while deficiency of conventional calpains significantly attenuated the development of atherosclerotic lesions[68]. Thus, it is suspected that vascular component cells, such as ECs or VSMCs, or other immune cells are involved in cal-

pain-dependent aortic aneurysms.

Diabetic Angiopathy

Type 1 and type 2 diabetes mellitus are chronic metabolic diseases caused by impaired insulin production in the pancreas and systemic insulin resistance, respectively, leading to the dysregulation of plasma glucose levels. It is well known that hyperglycemia results in vascular disorders, including diabetic retinopathy and nephropathy, which are the major complications of this disease[5]. Among the vascular component cells, dysfunction of ECs is primarily responsible for hyperglycemia-induced angiopathy[69]. While the exact mechanisms underlying these endothelial disorders are still unclear, the majority of studies have been based on the context that endothelial disorders are caused by excessive oxidative stress in blood vessels[70]. Indeed, it was reported that the exposure of ECs to high glucose conditions resulted in the elevation of ROS levels[71]. Furthermore, endothelial-dependent vasodilation, which is measured as an index of endothelial integrity, was reduced in diabetic animals, and was recovered by the administration of antioxidants[72]. Indeed, the elevation of ROS levels in blood vessels was detected in diabetic mice and human diabetic patients[73, 74], suggesting a pivotal role of ROS signaling in the pathogenesis of diabetic endothelial disorders. It was previously reported that the major source of ROS in blood vessels is NADPH oxidase, xanthine oxidase and uncoupled nitric oxide (NO) synthase (NOS) as well as mitochondrial respiration[75]. Uncoupled eNOS, which generates superoxides instead of NO, as well as xanthine oxidase, and NADPH oxidase are largely responsible for ROS production even in diabetic vessels[76, 77]. It was reported that physiological NO production in ECs was dependent upon conventional calpains. Youn *et al*. noted that the vascular endothelial growth factor (VEGF)-induced production of NO in cultured ECs was reduced by calpain inhibitors ALLN (N-acetyl-leucyl-leucyl-norleucinal) or calpeptin[78]. VEGF facilitates calpain translocation to the plasma membrane and formation of the molecular complex together with ezrin; as a result, the calpain-associated molecular complex potentiates the phosphorylation of AKT, AMP-dependent kinase and endothelial NOS (eNOS)$_{s1179}$, which is necessary for VEGF-induced NO production and subsequent angiogenic responses in ECs. Conversely, calpain-induced defects of endothelial NO systems have been also proposed. Cell-based experiments showed that ionophore-induced calpain overactivation proteolyzed heat shock protein 90 (HSP90) as well as eNOS and neural NOS[79]. Whereas the difference between the VEGF- and ionophore-induced NO production has not been thoroughly elucidated, Ca^{2+} overloading in cytoplasm might cause the excessive activation of conventional calpains thereby degrading unusual non-physiologic substrates including HSP90, which seems to be distinct from the physiological regulation mechanism. In addition to physiologic NO production, calpain was reportedly associated with defective NO systems in diabetes. Stalker *et al*. previously reported that inhibition of conventional calpains by ZLLal (aldehyde benzyloxycarbonyl-leucyl-leucinal) recovers impaired NO production and subsequent vasculitis in mesenteric venules in ZDF rats, concomitantly with the increasing association of eNOS with HSP90[80]. Chen *et al*. reported that conventional calpains in human umbilical vein endothelial cells were activated under high glucose conditions, accompanied by a reduction of NO production and elevation of cytosolic ROS levels without altering NOS expression levels[81]. Intriguingly, the glucose-induced changes in NO and ROS production were reversed by the transduction of calpastatin. Furthermore, aortic ROS levels were elevated in transgenic type 1 diabetic OVE26 mice, and were inhibited by calpastatin transduction. While endothelium-dependent vasodilation in diabetic OVE26 mice was blunted, calpastatin transduction improved the endothelial integrity of diabetic animals[81]. It was also documented that siRNA against calpain-1 prevented phosphorylation of eNOS at threonine 497/495 in murine aorta and cultured ECs under the hyperhomocysteinemia/high glucose conditions thereby improving NO production and subsequent EC integrity[82]. Thus, the overactivation of conventional calpain systems under diabetic conditions promotes dysfunctional endothelial NO systems, leading to reduction of endothelial integrity.

In addition to its defective regulation of NO signaling, the calpain-induced perturbation of prostanoid signaling has been reported as another pathogenic cue for diabetic angiopathy. Randriamboavonjy *et al*. reported that pharmacological calpain inhibition by A-705232 recovered EC-dependent vasodilation in diabetic mesenteric arteries[83]. This recovery was dependent upon protection by prostaglandin I_2 synthase (PGIS) from calpain-induced degradation. While the treatment of mesenteric arteries with peroxynitrate donors impaired endothelium-dependent vasodilation even in the presence or absence of a NO scavenger[83], it was recovered by the calpain inhibitor calpeptin. Collectively, the overactivation of conventional calpains opposes prostaglandin I_2 synthesis in diabetic small vessels independent of NO signaling, which might cause diabetic angiopathy.

Proliferative Retinopathy and Cancer Neovessels

It is well known that abnormalities of pathologi-

cal neovessels in proliferative retinopathy and tumor neovessels are caused by defective angiogenic signaling in ECs. During normal physiological status, ECs rearrange their shape via oxygen sensors and hypoxia-inducible factors, including prolyl hydroxylase domain 2 and hypoxia-inducible factor (HIF)-2α, respectively to optimize blood flow[84]. When the tissue becomes hypoxic, angiogenic responses are robustly activated through angiogenic mediators including VEGF-A, VEGF-C, fibroblast growth factors and angiopoietin-2. Such mediators accelerate the motility and mitosis of leading ECs (termed the tip cell) mainly through their surface receptors thereby driving endothelial tube formation. Tip cells guide the following ECs (termed stalk cells) and VEGFR2 in stalk cells is subsequently downregulated through the DLL4/NOTCH pathway to facilitate the coverage of immature vessels with pericytes[84]. According to such stepwise vascular maturation, the physiological neovessels acquire a hierarchical network as shown in the pre-existing vasculature. Increasing evidence suggests that conventional calpain systems contribute to physiological angiogenesis. For example, Zheng *et al.* recently noted the calpain-induced acceleration of HIF-1 signals[85]. In this case, calpain-induced the proteolysis of filamin-A, a large cytoskeletal actin-binding protein, whereby filamin-A proteolytic fragments physically interacted with HIF-1α to promote the nuclear localization of HIF-1α. However, it is noteworthy that *Capn1*-deficient mice as well as EC-specific *Capn2*-deficient mice did not exhibit the lethal vascular defects[23, 26]; thus, the impact of calpain-dependent angiogenesis on physiological vascular regulations is currently unclear. Please refer to an excellent review for further details of calpain-mediated physiological angiogenesis[86].

In contrast to the physiological neovessels, pathological neovessels, which are frequently detected in inflamed lesions (e.g. atherosclerotic lesions and diabetic retinopathy) as well as tumor tissues, exhibit an unstable and non-hierarchical structure[84, 87, 88]. Although both physiological and pathological angiogenesis are similarly driven by common angiogenic cues (e.g. angiopoietin-2 and VEGF), pathological neovessels exhibit immature properties characterized by a lack of pericyte coverage, a thin extracellular matrix layer and poor inter-endothelial junctions[84]. Because of these structural instabilities, pathological neovessels provide a major route for the recruitment of immune cells into inflamed lesions or for metastatic cancer cell emigration from primary tumor tissues, which leads to the further progression of disease. The critical difference between physiological and pathological angiogenesis is that the latter is affected by the surrounding inflammatory conditions. Interestingly, inflammatory and angiogenic signals are slightly interrelated and redundant. Indeed, several classes of proinflammatory cytokines, including TNF-α and IL-6, have angiogenic effects on vascular ECs[89-91]. Furthermore, the ablation of endothelial SOCS3, an endogenous inhibitor of JAK/STAT cytokine signals, accelerated growth factor- and cytokine-induced angiogenesis thereby aggravating cancer growth and oxygen-induced retinopathy (OIR) in mice[90]. It was reported that conventional calpains are associated with pathological angiogenesis. Indeed, Hoang *et al.* noted that calpain inhibitors MDL 28170, PD150606, or ALLN opposed the pathogenesis of OIR in mice, and normalized the morphology of retinal vessels[92]. Furthermore, we previously documented the contribution of endothelial calpains to pathological angiogenesis[35]. Mechanistically, the loss of calpastatin by several growth factor classes, causes the calpain-1-induced proteolytic degradation of SOCS3, leading to VEGF-C production through excessive JAK/STAT signaling. Accordingly, calpastatin downregulation facilitates IL-6-driven angiogenic responses in ECs through the local autocrine action of VEGF-C. Similarly, the downregulation of calpastatin and SOCS3 expression is detectable in neovessels in human colon adenocarcinoma, lung adenocarcinoma and malignant astrocytoma. The EC-specific transduction of calpastatin counteracts the STAT3/VEGF-C axis and antagonizes pathological angiogenesis in allograft tumors and OIR in mice, which suppresses such diseases. Whereas pathological neovessels in these diseases lack coverage of pericytes and formation of a basement membrane, calpastatin transduction improves the maturity of neovessels. Therefore, the overactivation of conventional calpains sensitizes JAK/STAT inflammatory cascades, which converts angiogenic ECs to pathological status.

In addition to angiogenic roles in ECs, conventional calpains are associated with the metabolism of angiostatic mediators. Saito *et al.* reported that calpain in cancer cells proteolytically degraded the angiostatic peptide vasohibin-1[33]. Vasohibin-1 synthesized in ECs can inhibit angiogenic responses by an autocrine mechanism; thus, the degradation of vashibin-1 by tumoral calpains might facilitate tumor angiogenesis.

Vascular Fibrosis and Pulmonary Hypertension

It is well known that aging induces structural and functional changes in arteries, and is a critical risk factor for cardiovascular defects, such as hypertension and atherosclerosis[93, 94]. An age-associated increase in arterial wall stiffness is largely dependent upon remodeling of the ECM and vascular calcification. In general, ECM remodeling in aged arteries occurs because of increased collagen content, elastin fragmentation, and is triggered by the transformation of contractile VSMCs to a synthetic phenotype. Letavernier *et al.*

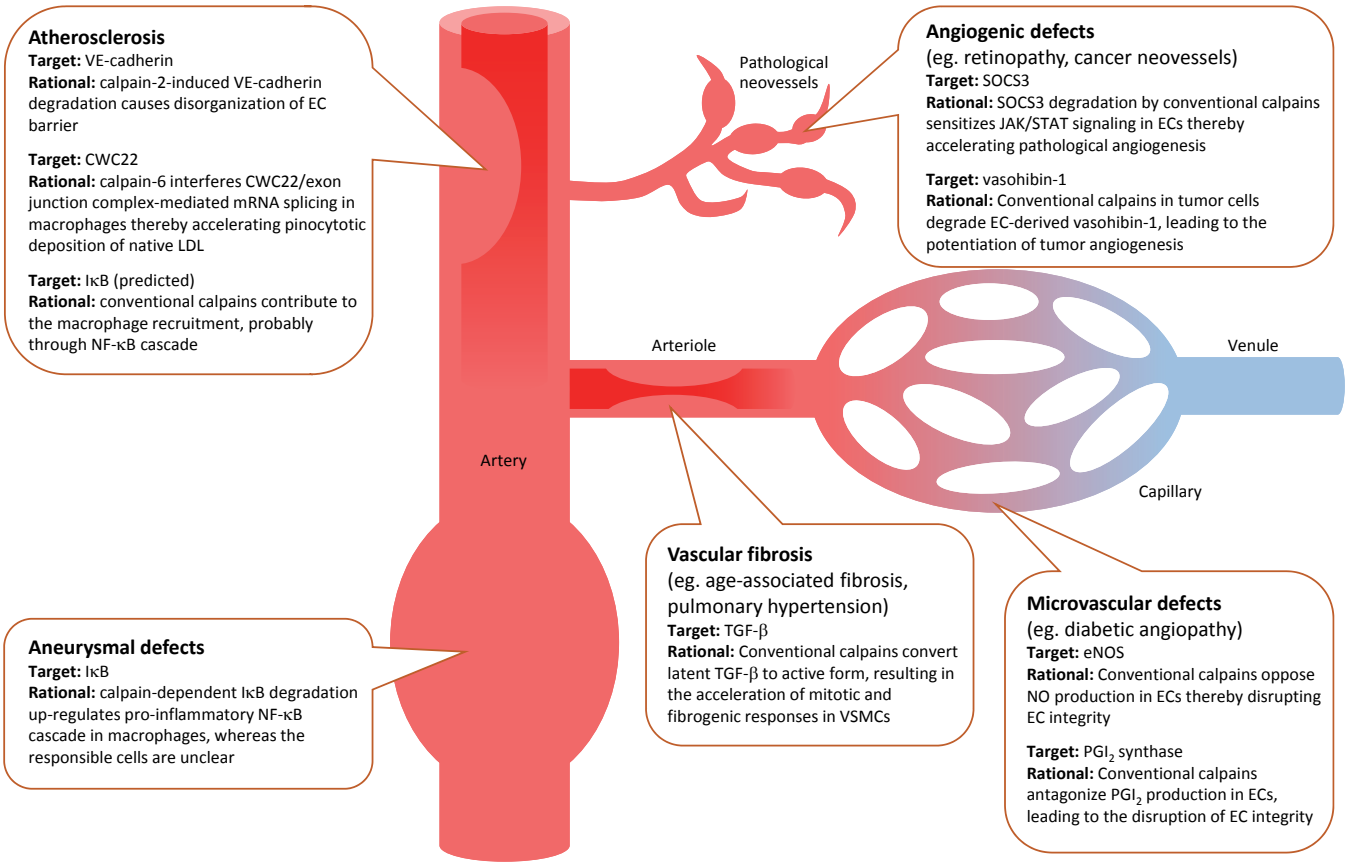

Fig. 2. Overview of the regulation of degenerative vascular disorders by calpain systems. LDL: low-density lipoprotein, EC: vascular endothelial cells, VSMCs: vascular smooth muscle cells, NFκB: nuclear factor κB, IκB: inhibitor κB, SOCS3: suppressor of cytokine signalling 3, JAK/STAT: Janus kinase/signal transducer and activator of transcription, NOS: nitric oxide synthase.

reported that vascular fibrosis and hypertrophy in angiotensin II-infused mice was prevented by calpastatin transduction[95]. Furthermore, impaired fibrogenic responses were caused by a reduction of medial MMP levels and NF-κB activity. Similarly, Jiang *et al.* reported that the induction of calpain-1 in VSMCs was associated with MMP2 upregulation and subsequent age-associated vascular fibrosis in rats[19]. Furthermore, the overexpression of calpain-1 in rats accelerated arterial calcification as well as fibrosis in aged rats, concomitant with a reduction of osteopontin and osteonectin levels in VSMCs[96]. Tang *et al.* reported that the transduction of calpastatin reduced VSMC proliferation and collagen synthesis thereby inhibiting restenosis induced by carotid artery ligation in mice[97]. Thus, calpain systems contribute to the variety of fibrogenic and proliferative responses in VSMCs.

Pulmonary hypertension is a proliferative vascular disease characterized by medial thickening and severe fibrosis in the right ventricular artery thereby increasing pulmonary vascular resistance and subsequent right heart failure[98, 99]. Excessive proliferation of VSMCs and hypertrophy as well as the production of ECM contributes to medial hypertrophy, leading to the destruction of precapillary pulmonary arteries and a sustained elevation of pulmonary arterial pressure. Ma *et al.* previously reported that a deficiency of *Capns1* reduced both calpain-1 and -2 levels, reduced collagen synthesis and remodeling in pulmonary arterioles and the subsequent pathogenesis of pulmonary hypertension in mice[100]. Calpain activated transforming growth factor (TGF)-β1 via direct proteolytic processing; thus, calpain deficiency resulted in the prevention of TGF-β1-induced fibrogenic responses in VSMCs. They also reported that calpain-2 activated Akt via the TGF-β1-mTORC2 pathway in pulmonary artery smooth muscle cells[101]. However, patients suffering from idiopathic pulmonary arterial hypertension exhibited high plasma calpastatin levels[102]. The hepatocyte-specific transduction of calpastatin reduced pulmonary calpain activity, resulting in the amelioration of murine hypoxia-induced pulmonary hypertension[102]. Thus, reduced extracellu-

Table 1. Possible contribution of calpain systems in degenerative vascular disorders.

Disease type	Experimental strategy	Intervention	Target molecule	Outcomes	Ref.
Atherosclerosis	*Apoe*$^{-/-}$ mice + high fat diet	Calpeptin	VE-cadherin	Atherosclerotic lesion↓	34
	Ldlr$^{-/-}$ mice + high fat diet	ALLM		Aortic macrophage recruitment↓	
				Endothelial permeability↓	
	Human umbilical vein endothelial cells + lysophosphatidylcholine	*Capn2* siRNA	VE-cadherin	Endothelial permeability↓	34
				VE-cadherin proteolysis↓	
	Ldlr$^{-/-}$ mice + high fat diet	*Capn6*$^{-/-}$	CWC22	Atherosclerotic lesion↓	36
				Aortic macrophage recruitment↓	
				Pinocytosis activity↓	
	Bone marrow-derived macrophages + TNF-α	*Capn6*$^{-/-}$	CWC22	Cellular motility↑	36
				Pinocytosis activity↓	
				Rac1 activity↑	
				Nuclear localization of CWC22↑	
	Ldlr$^{-/-}$ mice + high fat diet + AngII	BDA-410	–	Atherosclerotic lesion↓	57
	Ldlr$^{-/-}$ mice + high fat diet + AngII	*CAST*$^{+/0}$ (myeloid specific)	–	Atherosclerotic lesion↓	58
		Capn1$^{-/-}$ (myeloid specific)			
		Capn2$^{flox/flox}$/*LysM-Cre*$^{+/0}$			
	Ldlr$^{-/-}$ mice + high fat diet	*CAST*$^{+/0}$	–	Atherosclerotic lesion↓	58
Abdominal aortic aneurysm	*Ldlr*$^{-/-}$ mice + high fat diet + AngII	BDA-410	–	Abdominal aneurysm↓	57
				Aortic macrophage recruitment↓	
				MMP12↓	
				Proinflammatory genes↓	
	Ldlr$^{-/-}$ mice + high fat diet + AngII	*CAST*$^{+/0}$ (myeloid specific)	–	Abdominal aneurysm→	58
		Capn1$^{-/-}$ (myeloid specific)		Aortic macrophage recruitment↓	
		Capn2$^{flox/flox}$/*LysM-Cre*$^{+/0}$			
	Bone marrow-derived macrophages	*CAST*$^{+/0}$	IκBα	NF-κB cascade↓	58
Diabetic angiopathy	Zucker diabetic fatty rats (type 2 diabetes)	ZLLal	eNOS	NO production↑	80

(Cont Table 1)

Disease type	Experimental strategy	Intervention	Target molecule	Outcomes	Ref.
				EC-leukocyte interaction↓	
	Human umbilical vein endothelial cells + high glucose	Cast transduction	−	Nitric oxide production↓	81
		MDL28170		ROS production↓	
	OVE26 type 1 diabetic mice	*CAST*$^{+/0}$	−	Aortic ROS production↓	81
				EC-dependent vasodilation↑	
	Hyperhomocysteinemic/diabetic mice (cystathionine β-synthase-deficient mice + high-methionine diet + streptozotocin)	MDL28170	−	EC-dependent vasodilation↑	82
		Calpeptin		ROS production↓	
		ALLM			
	Human aortic endothelial cells	MDL28170	−	NO production↑	82
		CAPN1 siRNA		P-eNOS-pThr495↓	
	Streptozocin-induced diabetic mice	A-705232	PGI_2 synthase	EC-dependent vasodilation↑	83
	Diabetic New Zealand obese mice	Calpeptin		PGI_2 synthesis↑	
Pathological angiogenesis	Oxygen-induced retinopathy model in mice	MDL 28170	−	Oxygen-induced retinopathy↓	92
		PD150606		Retinal vascular permeability↓	
		ALLN			
	Oxygen-induced retinopathy model in mice	*LNL-CAST*$^{+/0}$/*Tie2-Cre*$^{+/0}$	SOCS3	Tumor angiogenesis↓	35
				Oxygen-induced retinopathy↓	
	Tumor implantation in mice			VEGF-C production↓	
				Maturation of neovessels↑	
	Human aortic endothelial cells + IL-6	*Cast* siRNA	SOCS3	Migration↑	35
				Proliferation↑	
				Tube formation↑	
				JAK/STAT3 signal↑	
				VEGF-C production↑	
	Co-culture B16 melanoma cells with ECs	MDL28170	Vasohibin-1	Vasohibin-1 proteolysis↓	33
Age-associated fibrosis	AngII-induced aortic hypertrophy model	*CAST*$^{+/0}$	−	Aortic fibrosis↓	95
				Aortic hypertrophy↓	
				Perivascular leukocyte recruitment↓	

(Cont Table 1)

Disease type	Experimental strategy	Intervention	Target molecule	Outcomes	Ref.
				NF-κB cascade↓	
	Primary VSMCs from aged rats + AngII	Calpain inhibitor I	−	MMP2 activity↓	19
		CAST transduction		VSMC migration↓	
	Primary VSMCs from aged rats	*CAPN1* transduction	−	Fibrosis↑	96
	Cultured carotid artery rings			Calcification↑	
				Smad1/2↑	
				TGF-β1↑	
				Osteopontin↓	
				Osteonectin↓	
Arterial restenosis	Carotid artery ligation model in mice	$CAST^{+/0}$	−	Arterial restenosis↓	97
				Arterial fibrosis↓	
				Arterial hypertrophy↓	
				Arterial MMP2/TGF-β1↓	
	Primary VSMCs from mice + PDGF-BB	*Capn1* siRNA	−	Proliferation↓	97
		Capn2 siRNA		Migration↓	
Pulmonary hypertension	Chronic hypoxia-induced pulmonary hypertension model in mice	$Capn2^{flox/flox}$/$ER\text{-}Cre^{+/0}$	TGF-β1	Pulmonary hypertension↓	100
				Collagen I in pulmonary arterioles↓	
				Hypertrophy in pulmonary arterioles↓	
	Monocrotaline-induced pulmonary hypertension model in rats	MDL28170	−	Pulmonary hypertension↓	100
				Hypertrophy in pulmonary arterioles↓	
	Human pulmonary arterial smooth muscle cells + PDGF-BB	MDL28170	TGF-β1	Collagen I synthesis↓	100
		Capn1 siRNA		Proliferation↓	
		Capn2 siRNA		TGF-β1 production↓	
	Chronic hypoxia-induced pulmonary hypertension model in mice	$Capn2^{flox/flox}$/$ER\text{-}Cre^{+/0}$	−	P-Akt-S473 in pulmonary arterioles↓	101
				P-Akt-T308 in pulmonary arterioles↓	
	Human pulmonary arterial smooth muscle cells + PDGF-BB	MDL28170	−	Collagen I synthesis↓	101
		Capn2 siRNA		Proliferation↓	
				P-Akt-S473↓	
				P-Akt-T308↓	

(Cont Table 1)

Disease type	Experimental strategy	Intervention	Target molecule	Outcomes	Ref.
	Murine hypoxia-induced pulmonary hypertension model	*CAST* transduction (CMV promoter)	−	Pulmonary hypertension↓	102
				Hypertrophy in pulmonary arterioles↓	
	SM22-5HTT1mice	*CAST* transduction (CRP promoter)			
		PD150606			
	Murine pulmonary arterial smooth muscle cells + FBS, + PDGF-BB, + EGF	PD150606	−	Proliferation↓	102

Abbreviations: CAPN: calpain, CAST: calpastatin, AngⅡ: angiotensin Ⅱ, EC: endothelial cells, VSMCs: vascular smooth muscle cells, FBS: fetal calf serum, PDGF: platelet-derived growth factor, EGF: epidermal growth factor, ALLN: N-acetyl-leucyl-leucyl-norleucinal (Calpain inhibitor Ⅰ), ALLM: N-Acetyl-L-leucyl-L-leucyl-L-methioninal (Calpain inhibitor Ⅱ), ZLLal; aldehyde benzyloxycarbonyl-leucyl-leucinal, MMP: matrix metalloproteinase, SOCS3: suppressor of cytokine signalling 3, JAK/STAT: Janus kinase/signal transducer and activator of transcription, PGI_2: prostaglandin I_2, ROS reactive oxygen species.

lar calpastatin levels as well as calpain activity in pulmonary arterioles may contribute to the pathogenesis of pulmonary hypertension.

Conclusive Remarks

In conclusion, the dysregulation of calpain systems is responsible for a variety of degenerative vascular disorders (**Fig. 2**), while these calpain systems are not substantially associated with vascular regulation under physiological conditions as well as vascular development. It is likely that the excessive activity of conventional calpains can induce these disorders, which are commonly caused by exogenous stressors, such as inflammatory cytokines and growth factors. Furthermore, loss of the endogenous inhibitor calpastatin potentiates calpain activity in some instances. In particular, it is noteworthy that the calpain-induced dysfunctional integrity of ECs and fibrogenic responses in VSMCs were reproduced by multiple studies (**Table 1**). In contrast to conventional isozymes, calpain-6 contributes to atherogenesis through its interference of mRNA splicing in macrophages. This indicates that calpain-6 might be associated with other macrophage-related diseases. Importantly, the loss-of-function of calpains almost uniformly ameliorates degenerative disorders. Indeed, the transgenic overexpression of calpastatin as well as calpain inhibitors such as MDL28170 and BDA-410, are effective treatments for degenerative disorders, suggesting that calpain inhibitors are potentially promising for the treatment of chronic vascular diseases. Candidate compounds for inhibiting conventional calpains are currently being tested in clinical trials including those of neurodegenerative diseases[14]. These agents should be repositioned for cardiovascular fields in the future, while agents that inhibit calpain-6 are currently unavailable. The development of subtype selective inhibitors, in particular those targeting unconventional calpains, is indispensable for treating atherosclerosis and related diseases.

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Conflict of Interest

None.

References

1) Ross R: Atherosclerosis--an inflammatory disease. N Engl J Med, 1999; 340: 115-126
2) Weber C, Noels H: Atherosclerosis: current pathogenesis and therapeutic options. Nat Med, 2011; 17: 1410-1422
3) Davis FM, Rateri DL, Daugherty A: Abdominal aortic aneurysm: novel mechanisms and therapies. Curr Opin

Cardiol, 2015; 30: 566-573
4) Geraldes P, King GL: Activation of protein kinase C isoforms and its impact on diabetic complications. Circ Res, 2010; 106: 1319-1331
5) Singleton JR, Smith AG, Russell JW, Feldman EL: Microvascular complications of impaired glucose tolerance. Diabetes, 2003; 52: 2867-2873
6) Gariano RF, Gardner TW: Retinal angiogenesis in development and disease. Nature, 2005; 438: 960-966
7) Konior A, Schramm A, Czesnikiewicz-Guzik M, Guzik TJ: NADPH oxidases in vascular pathology. Antioxid Redox Signal, 2014; 20: 2794-2814
8) Steinhubl SR: Why have antioxidants failed in clinical trials? Am J Cardiol, 2008; 101: 14D-19D
9) Kikuchi K, Tanaka E, Murai Y, Tancharoen S: Clinical trials in acute ischemic stroke. CNS Drugs. 2014; 28: 929-938
10) Miyazaki T, Koya T, Kigawa Y, Oguchi T, Lei XF, Kim-Kaneyama JR, Miyazaki A: Calpain and atherosclerosis. J Atheroscler Thromb, 2013; 20: 228-237
11) Zhang Y, Liu NM, Wang Y, Youn JY, Cai H: Endothelial cell calpain as a critical modulator of angiogenesis. Biochim Biophys Acta. 2017; 1863: 1326-1335
12) Goll DE, Thompson VF, Li H, Wei W, Cong J: The calpain system. Physiol Rev, 2003; 83: 731-801
13) Ono Y, Sorimachi H: Calpains: an elaborate proteolytic system. Biochim Biophys Acta, 2012; 1824: 224-236
14) Ono Y, Saido TC, Sorimachi H: Calpain research for drug discovery: challenges and potential. Nat Rev Drug Discov, 2016; 15: 854-876
15) Scalia R, Gong Y, Berzins B, Zhao LJ, Sharma K: Hyperglycemia is a major determinant of albumin permeability in diabetic microcirculation: the role of mu-calpain. Diabetes, 2007; 56: 1842-1849
16) Miyazaki T, Honda K, Ohata H: m-Calpain antagonizes RhoA overactivation and endothelial barrier dysfunction under disturbed shear conditions. Cardiovasc Res, 2010; 85: 530-541
17) Miyazaki T, Taketomi Y, Saito Y, Hosono T, Lei XF, Kim-Kaneyama JR, Arata S, Takahashi H, Murakami M, Miyazaki A: Calpastatin counteracts pathological angiogenesis by inhibiting suppressor of cytokine signaling 3 degradation in vascular endothelial cells. Circ Res, 2015; 116: 1170-1181
18) Miyazaki T, Honda K, Ohata H: Requirement of Ca2+ influx- and phosphatidylinositol 3-kinase-mediated m-calpain activity for shear stress-induced endothelial cell polarity. Am J Physiol Cell Physiol, 2007; 293: C1216-C1225
19) Jiang L, Wang M, Zhang J, Monticone RE, Telljohann R, Spinetti G, Pintus G, Lakatta EG: Increased aortic calpain-1 activity mediates age-associated angiotensin Ⅱ signaling of vascular smooth muscle cells. PLoS One, 2008; 3: e2231
20) Subramanian V, Moorleghen JJ, Balakrishnan A, Howatt DA, Chishti AH, Uchida HA: Calpain-2 compensation promotes angiotensin Ⅱ-induced ascending and abdominal aortic aneurysms in calpain-1 deficient mice. PLoS One, 2013; 8: e72214
21) Mikosik A, Jasiulewicz A, Daca A, Henc I, Frąckowiak JE, Ruckemann-Dziurdzińska K, Foerster J, Le Page A, Bryl E, Fulop T, Witkowski JM: Roles of calpain-calpastatin system (CCS) in human T cell activation. Oncotarget, 2016; 7: 76479-76495
22) Wu Z, Chen X, Liu F, Chen W, Wu P, Wieschhaus AJ, Chishti AH, Roche PA, Chen WM, Lin TJ: Calpain-1 contributes to IgE-mediated mast cell activation. J Immunol, 2014; 192: 5130-5139
23) Azam M, Andrabi SS, Sahr KE, Kamath L, Kuliopulos A, Chishti AH: Disruption of the mouse μ-calpain gene reveals an essential role in platelet function. Mol Cell Biol, 2001; 21: 2213-2220
24) Dutt P, Croall DE, Arthur JS, Veyra TD, Williams K, Elce JS, Greer PA: m-Calpain is required for preimplantation embryonic development in mice. BMC Dev Biol, 2006; 6: 3
25) Arthur JS, Elce JS, Hegadorn C, Williams K, Greer PA: Disruption of the murine calpain small subunit gene, Capn4: calpain is essential for embryonic development but not for cell growth and division. Mol Cell Biol, 2000; 20: 4474-4481
26) Takano J, Mihira N, Fujioka R, Hosoki E, Chishti AH, Saido TC: Vital role of the calpain-calpastatin system for placental-integrity-dependent embryonic survival. Mol Cell Biol, 2011; 31: 4097-4106
27) Franco SJ, Rodgers MA, Perrin BJ, Han J, Bennin DA, Critchley DR, Huttenlocher A: Calpain-mediated proteolysis of talin regulates adhesion dynamics. Nat Cell Biol, 2004; 6: 977-983
28) Serrano K, Devine DV: Vinculin is proteolyzed by calpain during platelet aggregation: 95 kDa cleavage fragment associates with the platelet cytoskeleton. Cell Motil Cytoskeleton, 2004; 58: 242-252
29) Chan KT, Bennin DA, Huttenlocher A (2010) Regulation of adhesion dynamics by calpain-mediated proteolysis of focal adhesion kinase (FAK). J Biol Chem, 285: 11418-11426
30) Kishimoto A, Mikawa K, Hashimoto K, Yasuda I, Tanaka S, Tominaga M, Kuroda T, Nishizuka Y: Limited proteolysis of protein kinase C subspecies by calcium-dependent neutral protease (calpain). J Biol Chem, 1989; 264: 4088-4092
31) Milligan SA, Owens MW, Grisham MB: Inhibition of IκB-α and IκB-β proteolysis by calpain inhibitor I blocks nitric oxide synthesis. Arch Biochem Biophys, 1996; 335: 388-395
32) Lin YC, Brown K, Siebenlist U: Activation of NF-κB requires proteolysis of the inhibitor IκB-α: signal-induced phosphorylation of IκB-α alone does not release active NF-κB. Proc Natl Acad Sci USA, 1995; 92: 552-556
33) Saito M, Suzuki Y, Yano S, Miyazaki T, Sato Y: Proteolytic inactivation of anti-angiogenic vasohibin-1 by cancer cells. J Biochem, 2016; 160: 227-232
34) Miyazaki T, Taketomi Y, Takimoto M, Lei XF, Arita S, Kim-Kaneyama JR, Arata S, Ohata H, Ota H, Murakami M, Miyazaki A: m-Calpain induction in vascular endothelial cells on human and mouse atheromas and its roles in VE-cadherin disorganization and atherosclerosis. Circulation, 2011; 124: 2522-2532
35) Miyazaki T, Taketomi Y, Saito Y, Hosono T, Lei XF, Kim-Kaneyama JR, Arata S, Takahashi H, Murakami M, Miyazaki A: Calpastatin counteracts pathological angio-

genesis by inhibiting suppressor of cytokine signaling 3 degradation in vascular endothelial cells. Circ Res, 2015; 116: 1170-1181
36) Miyazaki T, Tonami K, Hata S, Aiuchi T, Ohnishi K, Lei XF, Kim-Kaneyama JR, Takeya M, Itabe H, Sorimachi H, Kurihara H, Miyazaki A: Calpain-6 confers atherogenicity to macrophages by dysregulating pre-mRNA splicing. J Clin Invest, 2016; 126: 3417-3432
37) Tonami K, Hata S, Ojima K, Ono Y, Kurihara Y, Amano T, Sato T, Kawamura Y, Kurihara H, Sorimachi H: Calpain-6 deficiency promotes skeletal muscle development and regeneration. PLoS Genet, 2013; 9: e1003668
38) Dear N, Matena K, Vingron M, Boehm T: A new subfamily of vertebrate calpains lacking a calmodulin-like domain: implications for calpain regulation and evolution. Genomics, 1997; 45: 175-184
39) Johnson JD, Han Z, Otani K, Ye H, Zhang Y, Wu H, Horikawa Y, Misler S, Bell GI, Polonsky KS: RyR2 and calpain-10 delineate a novel apoptosis pathway in pancreatic islets. J Biol Chem, 2004; 279: 24794-24802
40) Arrington DD, Van Vleet TR, Schnellmann RG: Calpain 10: a mitochondrial calpain and its role in calcium-induced mitochondrial dysfunction. Am J Physiol Cell Physiol, 2006; 291: C1159-C1171
41) Sreenan SK, Zhou YP, Otani K, Hansen PA, Currie KP, Pan CY, Lee JP, Ostrega DM, Pugh W, Horikawa Y, Cox NJ, Hanis CL, Burant CF, Fox AP, Bell GI, Polonsky KS: Calpains play a role in insulin secretion and action. Diabetes, 2001; 50: 2013-2020
42) Lynn S, Evans JC, White C, Frayling TM, Hattersley AT, Turnbull DM, Horikawa Y, Cox NJ, Bell GI, Walker M: Variation in the calpain-10 gene affects blood glucose levels in the British population. Diabetes, 2002; 51: 247-250
43) Baier LJ, Permana PA, Yang X, Pratley RE, Hanson RL, Shen GQ, Mott D, Knowler WC, Cox NJ, Horikawa Y, Oda N, Bell GI, Bogardus C: A calpain-10 gene polymorphism is associated with reduced muscle mRNA levels and insulin resistance. J Clin Invest, 2002; 106: R69-R73
44) Cheverud JM, Fawcett GL, Jarvis JP, Norgard EA, Pavlicev M, Pletscher LS, Polonsky KS, Ye H, Bell GI, Semenkovich CF: Calpain-10 is a component of the obesity-related quantitative trait locus Adip1. J Lipid Res, 2010; 51: 907-913
45) Sugimoto K, Katsuya T, Ishikawa K, Iwashima Y, Yamamoto K, Fu Y, Matsuo A, Motone M, Rakugi H, Ogihara T: UCSNP-43 G/A polymorphism of calpain-10 gene is associated with hypertension and dyslipidemia in Japanese population. Am J Hypertens, 2003; 16 (Suppl 1): A82
46) Hoffstedt J, Naslund E, Arner P: Calpain-10 gene polymorphism is associated with reduced beta(3)-adrenoceptor function in human fat cells. J Clin Endocrinol Metab, 2002; 87: 3362-3367
47) Orho-Melander M, Klannemark M, Svensson MK, Ridderstrale M, Lindgren CM, Groop L: Variants in the calpain-10 gene predispose to insulin resistance and elevated free fatty acid levels. Diabetes, 2002; 51: 2658-2664
48) Ordovas JM: Genetic links between diabetes mellitus and coronary atherosclerosis. Curr Atheroscler Rep, 2007; 9: 204-210
49) Ross R: Atherosclerosis--an inflammatory disease. N Engl J Med, 1999; 340: 115-126
50) Weber C, Noels H: Atherosclerosis: current pathogenesis and therapeutic options. Nat Med, 2011; 17: 1410-1422
51) Moore KJ, Sheedy FJ, Fisher EA: Macrophages in atherosclerosis: a dynamic balance. Nat Rev Immunol, 2013; 13: 709-721
52) Chang TY, Chang CC, Ohgami N, Yamauchi Y: Cholesterol sensing, trafficking, and esterification. Annu Rev Cell Dev Biol, 2006; 22: 129-157
53) Legein B, Temmerman L, Biessen EA, Lutgens E: Inflammation and immune system interactions in atherosclerosis. Cell Mol Life Sci, 2013; 70: 3847-3869
54) Komohara Y, Fujiwara Y, Ohnishi K, Shiraishi D, Takeya M: Contribution of macrophage polarization to metabolic diseases. J Atheroscler Thromb, 2016; 23: 10-17
55) Karasawa T, Takahashi M: Role of NLRP3 Inflammasomes in atherosclerosis. J Atheroscler Thromb, 2017; 24: 443-451
56) Giannotta M, Trani M, Dejana E: VE-cadherin and endothelial adherens junctions: active guardians of vascular integrity. Dev Cell, 2013; 26: 441-454
57) Subramanian V, Uchida HA, Ijaz T, Moorleghen JJ, Howatt DA, Balakrishnan A. Calpain inhibition attenuates angiotensin Ⅱ-induced abdominal aortic aneurysms and atherosclerosis in low-density lipoprotein receptor-deficient mice. J Cardiovasc Pharmacol, 2012; 59: 66-76
58) Howatt DA, Balakrishnan A, Moorleghen JJ, Muniappan L, Rateri DL, Uchida HA, Takano J, Saido TC, Chishti AH, Baud L, Subramanian V: Leukocyte calpain deficiency reduces angiotensin Ⅱ-Induced inflammation and atherosclerosis but not abdominal aortic aneurysms in mice. Arterioscler Thromb Vasc Biol, 2016; 36: 835-845
59) Steckelberg AL, Boehm V, Gromadzka AM, Gehring NH: CWC22 connects pre-mRNA splicing and exon junction complex assembly. Cell Rep, 2012; 2: 454-461
60) Miyazaki T, Miyazaki A: Emerging roles of calpain proteolytic systems in macrophage cholesterol handling. Cell Mol Life Sci, in press
61) Anderson RN: Deaths: leading causes for 2000. Natl Vital Stat Rep, 2002; 50: 1-85
62) Kigawa Y, Miyazaki T, Lei XF, Kim-Kaneyama JR, Miyazaki A: Functional heterogeneity of nadph oxidases in atherosclerotic and aneurysmal diseases. J Atheroscler Thromb, 2017; 24: 1-13
63) McCormick ML, Gavrila D, Weintraub NL: Role of oxidative stress in the pathogenesis of abdominal aortic aneurysms. Arterioscler Thromb Vasc Biol, 2007; 27: 461-469
64) Longo GM, Xiong W, Greiner TC, Zhao Y, Fiotti N, Baxter BT: Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms. J Clin Invest, 2002; 110: 625-632
65) Luttun A, Lutgens E, Manderveld A, Maris K, Collen D, Carmeliet P, Moons L: Loss of matrix metalloproteinase-9 or matrix metalloproteinase-12 protects apolipoprotein E-deficient mice against atherosclerotic media destruction but differentially affects plaque growth. Circulation, 2004; 109: 1408-1414

66) Pilop C, Aregger F, Gorman RC, Brunisholz R, Gerrits B, Schaffner T, Gorman JH 3rd, Matyas G, Carrel T, Frey BM: Proteomic analysis in aortic media of patients with Marfan syndrome reveals increased activity of calpain 2 in aortic aneurysms. Circulation, 2009; 120: 983-991
67) Subramanian V, Uchida HA, Ijaz T, Moorleghen JJ, Howatt DA, Balakrishnan A: Calpain inhibition attenuates angiotensin Ⅱ-induced abdominal aortic aneurysms and atherosclerosis in low-density lipoprotein receptor-deficient mice. J Cardiovasc Pharmacol, 2012; 59: 66-76
68) Howatt DA, Balakrishnan A, Moorleghen JJ, Muniappan L, Rateri DL, Uchida HA, Takano J, Saido TC, Chishti AH, Baud L, Subramanian V: Leukocyte calpain deficiency reduces angiotensin Ⅱ-induced inflammation and atherosclerosis but not abdominal aortic aneurysms in mice. Arterioscler Thromb Vasc Biol, 2016; 36: 835-845
69) Xu J, Zou MH; Molecular insights and therapeutic targets for diabetic endothelial dysfunction. Circulation, 2009; 120: 1266-1286
70) Giacco F, Brownlee M: Oxidative stress and diabetic complications. Circ Res, 2010; 107: 1058-1070
71) Cifarelli V, Geng X, Styche A, Lakomy R, Trucco M, Luppi P: C-peptide reduces high-glucose-induced apoptosis of endothelial cells and decreases NAD(P)H-oxidase reactive oxygen species generation in human aortic endothelial cells. Diabetologia, 2011; 54: 2702-2712
72) Leo CH, Hart JL, Woodman OLL: 3',4'-Dihydroxyflavonol reduces superoxide and improves nitric oxide function in diabetic rat mesenteric arteries. PLoS One, 2011; 6: e20813
73) Lau YS, Tian XY, Huang Y, Murugan D, Achike FI, Mustafa MR: Boldine protects endothelial function in hyperglycemia-induced oxidative stress through an antioxidant mechanism. Biochem Pharmacol. 2013; 85: 367-375
74) Guzik TJ, Mussa S, Gastaldi D, Sadowski J, Ratnatunga C, Pillai R, Channon KM: Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. Circulation, 2002; 105: 1656-1662
75) Nowak WN, Deng J, Ruan XZ, Xu Q: Reactive Oxygen Species Generation and Atherosclerosis. Arterioscler Thromb Vasc Biol, 2017; 37: e41-e52
76) Fatehi-Hassanabad Z, Chan CB, Furman BL: Reactive oxygen species and endothelial function in diabetes. Eur J Pharmacol, 2010; 636: 8-17
77) Rochette L, Lorin J, Zeller M, Guilland JC, Lorgis L, Cottin Y, Vergely C: Nitric oxide synthase inhibition and oxidative stress in cardiovascular diseases: possible therapeutic targets? Pharmacol Ther, 2013; 140: 239-257
78) Youn JY, Wang T, Cai H: An ezrin/calpain/PI3K/AMPK/eNOSs1179 signaling cascade mediating VEGF-dependent endothelial nitric oxide production. Circ Res, 2009; 104: 50-59
79) Averna M, Stifanese R, De Tullio R, Salamino F, Bertuccio M, Pontremoli S, Melloni E: Proteolytic degradation of nitric oxide synthase isoforms by calpain is modulated by the expression levels of HSP90. FEBS J, 2007; 274: 6116-6127
80) Stalker TJ, Gong Y, Scalia R: The calcium-dependent protease calpain causes endothelial dysfunction in type 2 diabetes. Diabetes, 2005; 54: 1132-1140
81) Chen B, Zhao Q, Ni R, Tang F, Shan L, Cepinskas I, Cepinskas G, Wang W, Schiller PW, Peng T: Inhibition of calpain reduces oxidative stress and attenuates endothelial dysfunction in diabetes. Cardiovasc Diabetol, 2014; 13: 88
82) Cheng Z, Jiang X, Pansuria M, Fang P, Mai J, Mallilankaraman K, Gandhirajan RK, Eguchi S, Scalia R, Madesh M, Yang X, Wang H: Hyperhomocysteinemia and hyperglycemia induce and potentiate endothelial dysfunction via μ-calpain activation. Diabetes, 2015; 64: 947-959
83) Randriamboavonjy V, Kyselova A, Elgheznawy A, Zukunft S, Wittig I, Fleming I: Calpain 1 cleaves and inactivates prostacyclin synthase in mesenteric arteries from diabetic mice. Basic Res Cardiol, 2017; 112: 10
84) Carmeliet P, Jain RK: Molecular mechanisms and clinical applications of angiogenesis. Nature, 2011; 473: 298-307
85) Zheng X, Zhou AX, Rouhi P, Uramoto H, Borén J, Cao Y, Pereira T, Akyürek LM, Poellinger L: Hypoxia-induced and calpain-dependent cleavage of filamin A regulates the hypoxic response. Proc Natl Acad Sci USA, 2014; 111: 2560-2265
86) Zhang Y, Liu NM, Wang Y, Youn J, Cai H: Endothelial cell calpain as a critical modulator of angiogenesis. Biochim Biophys Acta, 2017; 1863: 1326-1335
87) Doyle B, Caplice N: Plaque neovascularization and antiangiogenic therapy for atherosclerosis. J Am Coll Cardiol, 2007; 49: 2073-2080
88) Gariano RF, Gardner TW: Retinal angiogenesis in development and disease. Nature, 2005; 438: 960-966
89) Sainson RC, Johnston DA, Chu HC, Holderfield MT, Nakatsu MN, Crampton SP, Davis J, Conn E, Hughes CC: TNF primes endothelial cells for angiogenic sprouting by inducing a tip cell phenotype. Blood, 2008; 111: 4997-5007
90) Stahl A, Joyal JS, Chen J, Sapieha P, Juan AM, Hatton CJ, Pei DT, Hurst CG, Seaward MR, Krah NM, Dennison RJ, Greene ER, Boscolo E, Panigrahy D, Smith LE: SOCS3 is an endogenous inhibitor of pathologic angiogenesis. Blood, 2012; 120: 2925-2929
91) Nilsson MB, Langley RR, Fidler IJ: Interleukin-6, secreted by human ovarian carcinoma cells, is a potent proangiogenic cytokine. Cancer Res, 2005; 65: 10794-10800
92) Hoang MV, Smith LE, Senger DR: Calpain inhibitors reduce retinal hypoxia in ischemic retinopathy by improving neovascular architecture and functional perfusion. Biochim Biophys Acta, 2011; 1812: 549-557
93) Lakatta EG: Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part Ⅲ: cellular and molecular clues to heart and arterial aging. Circulation, 2003; 107: 490-499
94) Nagai Y, Metter EJ, Earley CJ, Kemper MK, Becker LC, Lakatta EG, Fleg JL: Increased carotid artery intimal-medial thickness in asymptomatic older subjects with exercise-induced myocardial ischemia. Circulation, 1998; 98: 1504-1509
95) Letavernier E, Perez J, Bellocq A, Mesnard L, de Castro Keller A, Haymann JP, Baud L: Targeting the calpain/

calpastatin system as a new strategy to prevent cardiovascular remodeling in angiotensin Ⅱ-induced hypertension. Circ Res, 2008; 102: 720-728
96) Jiang L, Zhang J, Monticone RE, Telljohann R, Wu J, Wang M, Lakatta EG: Calpain-1 regulation of matrix metalloproteinase 2 activity in vascular smooth muscle cells facilitates age-associated aortic wall calcification and fibrosis. Hypertension, 2012; 60: 1192-1199
97) Tang L, Pei H, Yang Y, Wang X, Wang T, Gao E, Li D, Yang Y, Yang D: The inhibition of calpains ameliorates vascular restenosis through MMP2/TGF-β1 pathway. Sci Rep, 2016; 6: 29975
98) Rabinovitch M: Molecular pathogenesis of pulmonary arterial hypertension. J Clin Invest, 2012; 122: 4306-4313
99) Rabinovitch M: Pathobiology of pulmonary hypertension. Annu Rev Pathol, 2007; 2: 369-399
100) Ma W, Han W, Greer PA, Tuder RM, Toque HA, Wang KK, Caldwell RW, Su Y: Calpain mediates pulmonary vascular remodeling in rodent models of pulmonary hypertension, and its inhibition attenuates pathologic features of disease. J Clin Invest, 2011; 121: 4548-4566
101) Abeyrathna P, Kovacs L, Han W, Su Y: Calpain-2 activates Akt via TGF-β1-mTORC2 pathway in pulmonary artery smooth muscle cells. Am J Physiol Cell Physiol, 2016; 311: C24-34
102) Wan F, Letavernier E, Abid S, Houssaini A, Czibik G, Marcos E, Rideau D, Parpaleix A, Lipskaia L, Amsellem V, Gellen B, Sawaki D, Derumeaux G, Dubois-Randé JL, Delcroix M, Quarck R, Baud L, Adnot S: Extracellular Calpain/Calpastatin Balance Is Involved in the Progression of Pulmonary Hypertension. Am J Respir Cell Mol Biol, 2016; 55: 337-351