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 lamina. 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 I2, 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

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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. 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. 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; thus, many researchers have tested anti-oxidative agents in clinical trials. Unfortunately, these antioxidative trials have thoroughly failed to reverse chronic vascular diseases, although they were effective in acute inflammation, such as the acute phase of stroke.

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 Ca2+-dependent intracellular proteases and a calpain endogenous inhibitor, critically contribute to degenerative disorders. Calpains can be potentiated in the presence of pathophysiologic stressors, such as inflammatory cytokines, growth factors, bioactive lipids and hypoxic stimulus. 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.
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$^{13,21}$, 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$^{25}$; 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 Capn1 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 β (Iβ)$^{31,32}$ have been described previously. It was documented that conven-
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. 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, it is difficult to precisely define the cause of atherosclerosis; however, the major atherogenic risk factors include dyslipidemia and high free fatty acid levels in a Japanese population, obesity in a Scandinavian population, and free fatty acid levels in a Finnish population. It is likely that genetic variation in the CAPN10 locus confers higher cardiovascular disease risk to type 2 diabetes mellitus patients. Therefore, calpain-10 might induce diabetic angiopathy through its diabetogenic actions, whereas the direct role of this molecule in vascular regulation is unknown.

Calpain Regulates Vascular Degeneration

Calpain-10 is ubiquitously expressed and is associated with apoptosis in pancreatic islet cells, mitochondrial dysfunction, insulin secretion from pancreatic islets, and the oxidative utilization of glucose in skeletal muscle. **Capn10** deficiency in the SM/J mouse strain ameliorated insulin resistance and reduced blood glucose levels. 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; obesity in a Scandinavian population; and free fatty acid levels in a Finnish population. It is likely that genetic variation in the **CAPN10** locus confers higher cardiovascular disease risk to type 2 diabetes mellitus patients. Therefore, calpain-10 might induce diabetic angiopathy through its diabetogenic actions, whereas the direct role of this molecule in vascular regulation is unknown.
Inflammatory ability. Furthermore, inflammatory production of interleukin (IL)-1β reportedly facilitates development of atherosclerotic lesions; 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. 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. 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. Subramanian et al. also noted that atherosclerotic lesion progression in angiotensin II-infused Ldlr−/− mice was impaired by the calpain inhibitor BDA-410. Transgenic overexpression of calpastatin significantly attenuated angiotensin II- or hypercholesterolemia-induced atherosclerotic development in Ldlr−/− mice. Furthermore, myeloid calpain-1 or leukocyte calpain-2 were involved in angiotensin II-induced atherosclerosis. 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. 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; 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 pinocytotic 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. 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. 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 and pro-atherogenic cholesterol handling in macrophages.

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. 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 disease. Indeed, macrophage- and vascular cell-derived MMP-9 and -12 are considered major exacerbators of aneurysmal disease, because they break down vascular structures, including the elastic lamellae and basement membrane, which leads to vascular dissection. Previous proteomic analyses demonstrated the contribution of calpain-2 to aortic aneurysms in Marfan syndrome; 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. 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. 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 disease75. Among the vascular components, dysfunction of ECs is primarily responsible for hyperglycemia-induced angiopathy59. 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 vessels76, 77. Indeed, it was reported that the exposure of endothelial cells to high glucose conditions resulted in the elevation of ROS levels71. Furthermore, endothelial-dependent vasodilatation, which is measured as an index of endothelial integrity, was reduced in diabetic animals, and was recovered by the administration of antioxidants78. Indeed, the elevation of ROS levels in blood vessels was detected in diabetic mice and human diabetic patients51-54, 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 respiration79. 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 vessels79, 80. 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 calpeptin78. 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)51, 57, 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 NOS79. Whereas the difference between the VEGF- and ionophore-induced NO production has not been thoroughly elucidated, Ca2+ 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 benzoxycarbonyl-leucyl-leucinal) recovers impaired NO production and subsequent vasculitis in mesenteric venules in ZDF rats, concomitantly with the increasing association of eNOS with HSP9080. 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 levels81. 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 animals82. 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 integrity83. 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-795232 recovered EC-dependent vasodilation in diabetic mesenteric arteries84. This recovery was dependent upon protection by prostaglandin I2 synthase (PGIS) from calpain-induced degradation. While the treatment of mesenteric arteries with peroxynitrite donors impaired endothelium-dependent vasodilation even in the presence or absence of a NO scavenger85, it was recovered by the calpain inhibitor calpeptin. Collectively, the overactivation of conventional calpains opposes prostaglandin I2 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 retain a mature phenotype characterized by an inability to undergo angiogenic transitions. However, during pathological conditions, such as inflammation, 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.

Intriguingly, inflammatory and angiogenic cytokine signals, accelerated growth factor- and cytokine-induced angiogenesis thereby aggravating cancer growth and oxygen-induced retinopathy (OIR) in mice. 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. Furthermore, we previously documented the contribution of endothelial calpains to pathological angiogenesis. 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. 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. 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.
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 [96]. 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 [97]. 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 [98]. 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 [99, 100]. 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 Capn1 reduced both calpain-1 and -2 levels, reduced collagen synthesis and remodeling in pulmonary arterioles and the subsequent pathogenesis of pulmonary hypertension in mice [101]. 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 [102]. However, patients suffering from idiopathic pulmonary arterial hypertension exhibited high plasma calpastatin levels [103]. The hepatocyte-specific transduction of calpastatin reduced pulmonary calpain activity, resulting in the amelioration of murine hypoxia-induced pulmonary hypertension [104]. Thus, reduced extracellular...
| Disease type                  | Experimental strategy                        | Intervention | Target molecule | Outcomes                              | Ref. |
|------------------------------|----------------------------------------------|--------------|----------------|---------------------------------------|------|
| Atherosclerosis              | *Apoe<sup>−/−</sup> mice + high fat diet      | Calpeptin    | VE-cadherin    | Atherosclerotic lesion↓               | 34   |
|                             | *Ldlr<sup>−/−</sup> mice + high fat diet     | ALLM         |                | Aortic macrophage recruitment↓       |      |
|                             |                                              |              |                | Endothelial permeability↓             |      |
|                             | Human umbilical vein endothelial cells + lysophosphatidylcholine | *Capa2 siRNA* | VE-cadherin    | Endothelial permeability↓             | 34   |
|                             |                                              |              |                | VE-cadherin proteolysis↓              |      |
|                             | *Ldlr<sup>−/−</sup> mice + high fat diet     | *Capn6<sup>−/−</sup>* | CWC22         | Atherosclerotic lesion↓               | 36   |
|                             |                                              |              |                | Aortic macrophage recruitment↓       |      |
|                             |                                              |              |                | Pinocytosis activity↓                 |      |
| Bone marrow-derived macrophages + TNF-α | *Capn6<sup>−/−</sup>* | CWC22       |                | Cellular motility↑                    | 36   |
| Abdominal aortic aneurysm    | *Ldlr<sup>−/−</sup> mice + high fat diet + Ang II | BDA-410      |                | Atherosclerotic lesion↓               | 57   |
| Diabetic angiopathy          | *Zucker diabetic fatty rats (type 2 diabetes)* | CAST<sup>+/+</sup> (myeloid specific) | LysM-Cre<sup>+/+</sup> | Abdominal aneurysm↓                   | 57   |
|                             |                                              | CAST<sup>+/+</sup> (myeloid specific) | LysM-Cre<sup>+/+</sup> | MMP12↓                               |      |
|                             |                                              | CAST<sup>+/+</sup> (myeloid specific) | LysM-Cre<sup>+/+</sup> | Proinflammatory genes↓                |      |
|                             |                                              | CAST<sup>+/+</sup> (myeloid specific) | LysM-Cre<sup>+/+</sup> | Abdominal aneurysm↓                   | 58   |
|                             |                                              | CAST<sup>+/+</sup> (myeloid specific) | LysM-Cre<sup>+/+</sup> | MMP12↓                               |      |
|                             |                                              | CAST<sup>+/+</sup> (myeloid specific) | LysM-Cre<sup>+/+</sup> | Proinflammatory genes↓                |      |
|                             |                                              | CAST<sup>+/+</sup> (myeloid specific) | LysM-Cre<sup>+/+</sup> | Abdominal aneurysm↓                   | 58   |
|                             |                                              | CAST<sup>+/+</sup> (myeloid specific) | LysM-Cre<sup>+/+</sup> | MMP12↓                               |      |
|                             |                                              | CAST<sup>+/+</sup> (myeloid specific) | LysM-Cre<sup>+/+</sup> | Proinflammatory genes↓                |      |
|                             |                                              | CAST<sup>+/+</sup> (myeloid specific) | LysM-Cre<sup>+/+</sup> | Abdominal aneurysm↓                   | 58   |
|                             |                                              | CAST<sup>+/+</sup> (myeloid specific) | LysM-Cre<sup>+/+</sup> | MMP12↓                               |      |
|                             |                                              | CAST<sup>+/+</sup> (myeloid specific) | LysM-Cre<sup>+/+</sup> | Proinflammatory genes↓                |      |
|                             |                                              | CAST<sup>+/+</sup> (myeloid specific) | LysM-Cre<sup>+/+</sup> | Abdominal aneurysm↓                   | 58   |
|                             |                                              | CAST<sup>+/+</sup> (myeloid specific) | LysM-Cre<sup>+/+</sup> | MMP12↓                               |      |
|                             |                                              | CAST<sup>+/+</sup> (myeloid specific) | LysM-Cre<sup>+/+</sup> | Proinflammatory genes↓                |      |
| Disease type | Experimental strategy | Intervention | Target molecule | Outcomes | Ref. |
|--------------|-----------------------|--------------|-----------------|----------|------|
| Human umbilical vein endothelial cells + high glucose | Cast transduction | – | Nitric oxide production | EC-leukocyte interaction↓ | 81 |
| OVE26 type I diabetic mice | MDL28170 | – | ROS production↓ | | |
| Hyperhomocysteinemic/diabetic mice (cystathionine β-synthase-deficient mice + high- methionine diet + streptozotocin) | MDL28170 | – | EC-dependent vasodilation↓ | | 82 |
| Human aortic endothelial cells | Calpeptin | MDL28170 | MDL28170 | ROS production↓ | 82 |
| Streptozocin-induced diabetic mice | Calpeptin | – | P-eNOS-pThr495↓ | | 83 |
| Diabetic New Zealand obese mice | Calpeptin | MDL 28170 | – | Oxygen-induced retinopathy↓ | 92 |
| Oxygen-induced retinopathy model in mice | Calpeptin | MDL 28170 | – | Retinal vascular permeability↓ | |
| Pathological angiogenesis | Calpeptin | MDL 28170 | – | | |
| Lung carcinoma model in mice | Calpeptin | PD150606 | – | | |
| Human aortic endothelial cells + IL-6 | Calpeptin | MDL 28170 | – | Migration↑ | 35 |
| Age-associated fibrosis | Calpeptin | MDL 28170 | – | | 95 |

This table continues from the previous page.
| Disease type                        | Experimental strategy | Intervention | Target molecule | Outcomes                      | Ref. |
|------------------------------------|-----------------------|--------------|----------------|-------------------------------|------|
| Primary VSMCs from aged rats + AngII | Calpain inhibitor 1   | —            | NF-κB cascade | MMP2 activity                 | 19   |
|                                    | CAST transduction     |              |                | VSMC migration                |      |
| Primary VSMCs from aged rats       | CAPN1 transduction    | —            | Fibrosis       |                               | 96   |
| Cultured carotid artery rings      |                       |              |                |                               |      |
| Arterial restenosis                | CAST<sup>+/−</sup>     | —            | Arterial restenosis |                               | 97   |
| Arterial fibrosis i                | Arterial hypertrophy i|              |                |                               |      |
| Arterial MMP2/TGF-β1 i             | Arterial MMP2/TGF-β1 i|              |                |                               |      |
| Primary VSMCs from mice + PDGF-BB  | Capn1 siRNA           | —            | Proliferation  |                               | 97   |
|                                    | Capn2 siRNA           |              | Migration      |                               |      |
| Pulmonary hypertension             | Capn1 siRNA           |              |                |                               |      |
| Monocrotaline-induced pulmonary hypertension model in mice | Capn2<sup>flo/flox/ER- Cre<sup>+/−</sup> | TGF-β1 | Pulmonary hypertension | Collagen I in pulmonary arterioles | 100 |
|                                    | MDL28170              | —            | Pulmonary hypertrophy | Hypertrophy in pulmonary arterioles | 100 |
| Human pulmonary arterial smooth muscle cells + PDGF-BB | MDL28170              | TGF-β1 | Collagen I synthesis | P-Akt-S473 in pulmonary arterioles | 101 |
|                                    | Capn1 siRNA           |              | Proliferation  | TGF-β1 production             |      |
|                                    | Capn2 siRNA           |              |                |                               |      |
| Chronic hypoxia-induced pulmonary hypertension model in mice | Capn2<sup>flo/flox/ER- Cre<sup>+/−</sup> | — | P-Akt-T308 in pulmonary arterioles | P-Akt-T308 in pulmonary arterioles | 101 |
| Human pulmonary arterial smooth muscle cells + PDGF-BB | MDL28170              | —            | Collagen I synthesis | P-Akt-S473 | 101 |
|                                    | Capn2 siRNA           |              | Proliferation  |                               |      |
calpain systems are currently being tested in clinical trials including those of neurodegenerative diseases\textsuperscript{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.

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(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              |
| SM22-5HTT1mice                                   | CAST transduction (CRP promoter)    | PD150606                          | –                | Hypertrophy in pulmonary arterioles | 102 |
| 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 1), ALLM: N-Acetyl-L-leucyl-L-leucyl-L-methioninal (Calpain inhibitor 2), ZLLLal; aldehyde benzoylcarbonyl-leucyl-leucinal, MMP: matrix metalloproteinase, SOCS3: suppressor of cytokine signalling 3, JAK/STAT: Janus kinase/signal transducer and activator of transcription, PGI: prostaglandin I, ROS reactive oxygen species.
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genetic suppressor of cytokine signaling 3 (SOCS-3) plays a role in the degradation of vascular endothelial growth factor receptor (VEGFR) and vascular endothelial growth factor (VEGF) in human umbilical vein endothelial cells (HUVECs) and smooth muscle cells (SMCs) in vitro. These findings suggest that SOCS-3 may be involved in the regulation of vascular growth and function. 

We further investigated the role of SOCS-3 in the regulation of cell proliferation and migration using siRNA-mediated silencing of SOCS-3. We found that down-regulation of SOCS-3 significantly increased cell proliferation and migration in HUVECs and SMCs. Moreover, we observed an increase in the expression of cyclin D1 and matrix metalloproteinase-2 (MMP-2) in SOCS-3-silenced cells, indicating that SOCS-3 may negatively regulate cell proliferation and migration via the up-regulation of these genes.

In conclusion, our study demonstrates that SOCS-3 plays a critical role in the regulation of vascular endothelial growth factor (VEGF) signaling and cell proliferation and migration in HUVECs and SMCs. These findings provide new insights into the molecular mechanisms underlying vascular growth and function and may have implications for the development of novel therapeutic strategies for cardiovascular diseases.
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