Despite many advances in percutaneous and surgical interventions in the treatment of coronary artery disease (CAD), up to one-third of patients are still either not candidates or receive suboptimal revascularization. Calpains are a class of calcium-activated non-lysosomal cysteine proteases that serve as a proteolytic unit for cellular homeostasis. Uncontrolled activation of calpain has been found to be involved in the pathogenesis of myocardial reperfusion injury, cardiac hypertrophy, myocardial stunning and cardiac ischemia. Inhibition of calpains has been shown to significantly attenuate myocardial stunning and reduced infarct size after ischemia-reperfusion. Calpain inhibition therefore serves as a potential medical therapy for patients suffering from a number of diseases, including CAD.  (Circ J 2016; 80: 4–10)

Key Words: Calpain; Coronary artery disease; Metabolic syndrome

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Calpain Structure
To date, 15 calpain isoforms have been reported. In mammals there are 14 large subunit members (80-kDa catalytic subunit), 1 small subunit member (30-kDa subunit) and 1 endogenous inhibitor (calpastatin). Both subunits can bind calcium, resulting in the activation of calpain. Calpain activity can also be regulated by auto proteolysis and by calpastatin, suggesting that calpains are part of a regulatory proteolytic system (Figure 2).

Most calpain isoforms contain 4 structural domains and calpains are divided into 2 groups based on the structure of domain IV: typical and atypical. Typical calpains (1, 2, 3, 8, 9, 11, 12, 14) contain a penta-EF motif in domain IV that can bind calcium, the calpain small subunit (only calpains 1, 2 and 9 have been shown to dimerize) or calpastatin. Atypical calpains (5, 6, 7, 10, 12 and 15) lack a penta-EF motif in domain IV and are unable to bind the calpain small subunit or calpastatin.

Some calpains are ubiquitously expressed (calpains 1, 2, 4, 5, 7 and 10), but others are found in specific tissues (calpain 1, 2: endothelia cells; calpain 3: skeletal muscle; calpain 6: placenta; calpain 8: smooth muscle; calpain 9: stomach; calpain 11: testes; calpain 12: skin after birth and calpain 13: testes and lung). Among the 15 calpain isoforms, 10 are expressed in the heart. The most well-studied of these are calpain 1 (U-calpain) and calpain 2 (m-calpain) which require micromolar concentrations of calcium for their activation, respectively.

In atypical calpains the function of domain 1 is largely unknown. However, domain 1 for calpain 10 contains a mitochondrial targeting sequence. In calpains 1, 2 and 9, domain I is cleaved after Ca2+ activation, resulting in autolysis and autoactivation. Domain II contains the catalytic active site with the “catalytic triad” of cysteine, asparagine and histidine. The catalytic triad is conserved throughout the entire family (except for...
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This fact is critical and should be taken into consideration when considering the use of calpain inhibitors for the treatment of cardiovascular disease.

CAD and MetS

It has been well demonstrated that the comorbid diseases that constitute MetS (ie, DM, obesity, and hypercholesterolemia) have a deleterious effect on endothelial function and growth. With the increasing prevalence of these comorbid conditions, it is important to understand the mechanisms by which these diseases contribute to or exacerbate endothelial dysfunction and CAD.

In particular, DM is recognized as a potent risk factor for calpain activity, which is required for normal protein homeostasis. This fact is critical and should be taken into consideration when considering the use of calpain inhibitors for the treatment of cardiovascular disease.
cardiovascular disease including atherosclerosis and microvascular complications.\textsuperscript{5,10} Hyperglycemia has been shown to cause endothelial dysfunction that is characterized by a loss of endothelium-derived nitric oxide (NO). Hyperglycemia also results in increased vascular permeability, increased endothelial adhesiveness, thickening of the basement membrane, abnormal inflammatory signals and increased oxidative stress. Endothelial NO is an important regulator of vascular homeostasis. Loss of NO leads to increased vascular tone, and abnormal endothelial adhesiveness, with resultant increased platelet aggregation and leukocyte trafficking in the vessel wall. Thus the diabetic vasculature experiences increased oxidative stress and abnormal inflammatory signals.\textsuperscript{10,11} Furthermore, high glucose-induced myocyte apoptosis plays an important role in diabetic cardiac complications. Though the molecular mechanisms by which glucose damages cardiac cells remain incompletely understood, it is thought to be partly mediated through cytopathic oxidative stress and intracellular calcium overload.\textsuperscript{12}

In order to further study the effect of these comorbidities on CAD, our group has utilized a clinically relevant large animal model that has been shown to adequately represent the metabolic, microcirculatory, and molecular abnormalities seen in MetS in adult patients with chronic myocardial ischemia.\textsuperscript{13} We have also demonstrated that MetS in chronic myocardial ischemia significantly impairs notch signaling (an integral component of cardiac angiogenesis) by downregulating notch receptors, ligands and angiogenic proteins.\textsuperscript{14} Expression of neuropeptide Y (one of the most abundant neurotransmitters in the myocardium known to influence cardiovascular remodeling) was found to be altered differentially in the serum and myocardium of patients with DM. Findings from our laboratory and others suggest that altered regulation of the neuropeptide Y in diabetic subjects may be, in part, responsible for the decreased angiogenesis, increased apoptosis and vascular smooth muscle proliferation leading to CAD and heart failure in the MetS patient population.\textsuperscript{15}

It is important to note that comorbid factors such as DM, obesity, and hypercholesterolemia will influence and exacerbate endothelial dysfunction leading to CAD. Understanding how these pathological conditions affect each other will help with devising therapeutics that would effectively treat heart disease. In the following section, we will discuss how calpain may be involved in the pathogenesis of DM, obesity and CAD, making it a potential therapeutic target.

Uncontrolled Activation of Calpains and Heart Disease

Calpain activity is required for normal myogenesis, whereas calpain overactivation has been implicated in the development of heart disease.\textsuperscript{2,16,17} In cardiac muscle, calpain plays a crucial role in excitation-contraction coupling. Dysregulation of cellular calcium homeostasis often results in calcium overload, which leads to calpain overactivation and results in myocardial cell injury.\textsuperscript{2} Uncontrolled activation of calpain has been found to be involved in the pathogenesis of myocardial reperfusion injury, cardiac hypertrophy, myocardial stunning and cardiac, cerebral and hepatocellular ischemia.\textsuperscript{18} Inhibition of calpains has been shown to significantly attenuate myocardial stunning and reduced infarct size after IR.\textsuperscript{5}

The exact mechanisms by which calpain is upregulated are not fully understood. Calpain activity is mainly regulated through alterations in the calcium concentration, as calcium binding is required for its proteolytic activity. Evidence has been provided that cellular calcium overload leads to overactivation of calpains by autoproteolysis of an N-terminal peptide.\textsuperscript{19} Studies also suggest that calpain activity can also be regulated by certain phospholipids: ERK1/2, mitogen-activated protein kinase, and protein kinases C and A. In addition, NADPH oxidase has been suggested to induce calpain activation in norepinephrine-stimulated cardiomyocytes. For example, one study suggested that the activation of NADPH oxidase results in reactive oxygen species (ROS) production, which activates calpain through regulation of intracellular calcium levels and the ERK1/2 pathway.\textsuperscript{9}

In concurrence with the foregoing discussion, our group and multiple others have demonstrated that overactivation of calpain contributes to cardiovascular disease.\textsuperscript{2,15,18,20} However, a better understanding of the proangiogenic and anti-angiogenic pathways in the setting of hypercholesterolemia, hyperglycemia, and chronic ischemic disease is necessary. Our laboratory has developed a pig model of chronic myocardial ischemia in the setting of MetS (weight gain, glucose intolerance, dyslipidemia and hypertension) to elucidate the molecular mechanisms in a preclinical setting. Further studies should focus on identifying the mechanisms by which this overactivation occurs and to identify novel therapies for calpain inhibition as a potential medical therapy for patient with cardiac risk factors and disease.

Calpains and Cytoskeletal Protein Remodeling

In diseased myocardial tissue, remodeling may be associated with ischemia-induced changes to the contractile proteins. Calpain has been found to cleave myofibrillar-specific proteins, including troponin T, troponin I, titin and desmin in cardiomyocytes.\textsuperscript{9} Cardiac troponin I is a subunit of the troponin complex involved in the control of muscle contraction. Interestingly, troponin I has been found to be preferentially affected in ischemic rat hearts.\textsuperscript{21} Maekawa et al. found that systolic dysfunction of the stunned myocardium is associated with troponin I degradation and overexpression of calpastatin will lessen the contractile dysfunction of rat hearts subjected to IR. In addition, the same model found that degradation of troponin following IR was prevented by calpain inhibition.\textsuperscript{22}

Yoshikawa et al. found that a high calcium intracoronary infusion caused cardiac overload-induced cardiac dysfunction through proteolysis of the cytoskeleton protein α-fodrin. Yoshikawa et al. found that calpain inhibition protected the rat heart from IR injury by inhibiting proteolysis of α-fodrin, which they speculated prevents conformational changes to the L-type calcium channel at the cell membrane and results in protection of left ventricular (LV) function.\textsuperscript{19}

Endothelial cells play an essential role in cardiovascular pathophysiology through their functions in blood vessel formation, cell barrier, coagulation, vascular tone, inflammation and angiogenesis. Many of these processes require cytoskeletal rearrangements and a number of cytoskeletal proteins are known calpain substrates, including talin, filamin, spectrin, tau, paxillin, vinculin, α-tubulin, vimentin, and laminin.\textsuperscript{5,7,9,10}

In vivo studies have demonstrated that calpain inhibitors strongly improved the organization of the endothelial actin and tau protein cytoskeleton in newly formed blood vessels, suggesting that calpain inhibition restores cytoskeletal organization, which improves capillary morphogenesis and promotes formation of a functionally improved neovascularure that helps to reduce underlying hypoxia.\textsuperscript{23,24}

Kudo-Sakamoto et al. demonstrated that calpains impaired cell-cell interactions through degradation of the cadherin-
associated protein complex, \(^5\) resulting in LV remodeling after myocardial infarction (MI). Interestingly, they found that calpain-mediated proteolysis was increased in the chronic phase (≥7 days) but not in the acute phase (<24 h) after MI, and profound activation of calpains exacerbated LV remodeling. The border zone of MI hearts in these mice showed a decrease in N-cadherin expression concomitant with an increase in calpain activation and exacerbation of LV remodeling. In cultured cardiomyocytes, calpain activation caused degradation of N-cadherin and disorganization of cadherin-based cell adhesions. \(^2\) Similarly, in cultured cardiomyocytes, calpain activation disassembled cadherin-based cell–cell adhesion consisting of intercalated disc proteins such as B-catenin and connexin 43. \(^2\) This suggests that calpain overactivation may lead to myocardial remodeling after MI through the breakdown of cell-cell interactions.

Additionally, several groups have reported roles for mitochondrial calpain 1 in endothelial cells. In rat heart microvascular endothelial cells, hyperhomocysteinemia induced extracellular matrix remodeling by matrix metalloproteinase 9 has been shown to be caused by calpain 1 overactivation. \(^6\)

Activation of calpain results in proteolysis of several cellular proteins, including cytoskeletal proteins that are known to play a role in myocardial tissue remodeling after an ischemic insult. This may be one mechanism by which calpain inhibition can help prevent ischemic changes in myocardial tissue after an MI.

### Calpains and Myocardial Apoptosis

Calpain plays an important role in cell death signaling in the heart. \(^3\) It has been documented that calpains are important in IR injury in the heart, mitochondrial permeability transition and necrotic/apoptotic cell death. \(^8\) Myocardial calpain 1 is active under normal physiological conditions and serves as an essential component of the ubiquitin-proteasome protein degradation pathway that removes proteins whose abnormal accumulation causes cardiomyocyte degeneration and heart failure. \(^7\) Excessive calpain activity has been shown to play a role in damaging mitochondria and oxidative phosphorylation during cardiac IR injury. \(^6\)

Many of the proteins involved in apoptotic signaling are substrates for activated calpain. Partial cleavage of pro-or anti-apoptotic proteins by calpain have been shown to activate or inactivate proteins such as caspases 3, 7, 8, 9, and 12, BLC-2, BCI-xl, Bid, Bax and nuclear factor κ-B (NF-κB). \(^20, 26\) Overexpression of calpastatin has been shown to abrogate caspase-3 activation and prevent apoptosis. \(^26\)

In the past few years, there has been growing evidence that supports a role for mitochondrial calpains in mitochondrial dysfunction. The mitochondrial permeability transition pore (mPTP) is a nonspecific pore located in the inner mitochondrial membrane that is permeable to small molecules. Opening of the mPTP during IR induces apoptosis through the mitochondrial release of proapoptotic proteins and ATP depletion. Inhibitors of mPTP opening limit the size of the MI in IR. mPTP opening is induced physiologically by calcium and is enhanced by mitochondrial calcium overload in IR. Shintani-Ishida and Yoshida demonstrated that mitochondrial matrix m-calpain contributes to complex I activation and mPTP opening after post-ischemic reperfusion in the rat heart. \(^27\)

Similarly, mitochondrial calpains have been reported to be proapoptotic proteases by mediating the cleavage of apoptosis inducing factor (AIF) after Ca\(^{2+}\) overload. AIF is bound to the inner mitochondrial membrane and when there is mitochondrial damage, AIF is cleaved, allowing it to translocate to the nucleus and induce DNA degradation. Smith et al showed that in the presence of exogenous Ca\(^{2+}\) in isolated heart cells, mitochondrial calpain causes activation of AIF. \(^6\) They also showed that pretreatment with MDL28170, a calpain inhibitor, prevents the release of AIF in isolated heart mitochondria, suggesting that inhibition of mitochondrial calpain 1 prevents the release of AIF, resulting in reduced cardiac cell death. \(^6\)

Another mechanism by which calpain induces cardiac cell injury is through proteolysis of protein kinase C, resulting in a constitutively active protein kinase M, which leads to stunned myocardium in IR injury. \(^22\) Specifically, it is thought that during oxidized-LDL-stimulated apoptosis, intracellular Ca\(^{2+}\) increases and induces calpain activation, which leads to Bid cleavage, cytochrome c release, apoptosis formation and caspase 3 activation. The increase in oxidized-LDL activation of calpain and induction of apoptosis in atherosclerotic areas suggests that this type of apoptosis is important in atherothrombotic events. \(^22, 27\)

Additional calpain substrates include the proapoptotic proteins p53 and transcription factors YY1, c-mos, c-jun and c-fos. \(^7, 16, 21\) Kubbutat et al found that calpain inhibits p53 to generate an N terminally truncated protein, and treatment of cells with a calpain inhibitor induced rapid accumulation of p53 protein, plausibly as a result of enhanced protein stability. \(^7\) Walowitz et al suggest that myogenic calpain activity functions through cleavage of the multifunctional transcription factor YY1, which is capable of repressing myogenic transcription via its DNA-binding activity. \(^16\) Interestingly, they found that calpain inhibition served to stabilize YY1 in myoblasts. Of particular importance is that calpain inhibition only stabilized YY1 in the setting of elevated calcium influx, \(^16\) which suggests that calpain overactivation could affect the transcription of genes important in cell death.

Oxidative stress in myocytes induces calpain activation, which in turn further increases oxidative stress. Studies indicate that hypoxia induces calpain activity in retinal, lung and umbilical vein endothelial cells. \(^23\) However, it is difficult to determine whether oxidative stress or calpain activation occurs first. Published results do suggest that calpain activation may promote oxidative stress, which in turn potentially leads to cellular apoptosis. \(^2, 20\) Future work is needed to elucidate the relationship between calpain activation and oxidative stress.

Inhibition of calpain overactivity has been reported to protect the myocardium against myocardial IR injury and cardiac hypertrophy by reducing infarct size, preventing LV remodeling and protecting LV function. \(^2, 5\) This suggests that calpain inhibition may be a good medical therapy for patients with CAD.

### Calpains and Angiogenesis in Ischemic Tissue

Moderate calpain inhibition in the setting of hypercholesterolemia and chronic myocardial ischemia has been found to improve proangiogenic protein expression, microvascular relaxation and myocardial perfusion. \(^3, 20\)

Vascular endothelial growth factor (VEGF)-A is essential for angiogenesis in a variety of important pathologies, including ischemia and wound repair, proliferative retinopathies, psoriasis, rheumatoid arthritis and cancers. Interestingly VEGF induces a highly abnormal vasculature in pathological settings and has been shown to induce calpain activity in endothelial cells. Khalil et al showed that, through VEGF, calpain inhibition reduced infarct size and improved the hemodynamics and contractile function in a large animal model of cardiac IR. \(^18\)

VEGF potentially works through induction of NO production
through PI3K/AKT and/or AMPK-dependent phosphorylation of endothelial NO synthase (eNOS). Exrin is a member of the exrin/radixin/moesin protein complex that is classically involved in cytoskeletal remodeling. Youn et al found that exrin-dependent membrane-specific translocation and activation of calpain by VEGF precedes AMPK- and AKT-dependent phosphorylation of eNOS and production of NO, suggesting a critical role for calpain interaction in modulating endothelial cell function.

Calpain activity has also been shown to induce hypoxic retina and calpain hyperactivation has been implicated in this retinal pathology. Hoang et al studied the relationship between VEGF and calpain both in vivo and in vitro. To determine the consequences of calpain inhibition on hypoxia-induced neovascularization, they used their established mouse model of ischemic retinopathy to illustrate that neovessels do not relieve hypoxia and that moderation of calpain activity offers a novel strategy for normalizing pathological retinal neovascularization and restoring normal oxygenation. They found that moderate calpain inhibition improved neovascular architecture and function, as measured by a reduction in abnormal vascular tufts and vascular leakage. Administration of calpain inhibitors also significantly improved vascular regrowth, as measured by improved vascular coverage of the retina. Most importantly, the improvement in neovascularization provided by calpain inhibitors resulted in marked reduction of underlying retinal hypoxia. In vivo studies found that calpain inhibitors strongly improved the organization of the endothelial actin and tau protein cytoskeleton in newly formed blood vessels. Their research suggests that calpain inhibition restores cytoskeletal organization, improves capillary morphogenesis and promotes formation of a functionally improved neovascular network that reduces underlying hypoxia. Notably, although appropriately moderate doses of calpain inhibitors improved vascular coverage in all cases, higher doses inhibited retinal neovascularization. In vitro capillary morphogenesis experiments indicated that optimal improvements were observed at 30–35% calpain inhibition (indicated by reduced formation of abnormal vascular tufts and focal leakage within 5 days of treatment). Complete suppression of calpain resulted in nonfunctional cells (indicated by cell rounding).

Stalker et al found that calpain activity is increased in the diabetic vasculature and that inhibition of calpain activity attenuates the vascular dysfunction associated with chronic DM. More specifically, they found that increased levels of endothelial NO and attenuated expression of ICAM-1 and VCAM-1 in diabetic rats. Inhibition of calpain activity restored the association of eNOS with hsp90, increased NO release and attenuated leukocyte trafficking in the microcirculation of non-insulin-dependent DM rats and that inhibition of calpain activity significantly attenuates leukocyte-endothelium interactions.

One of the cytokines released after IR injury is tumor necrosis factor (TNF)-α. Calpain has been implicated to play a role in TNF-α-mediated apoptosis in cardiomyocytes. It is thought that calpain activation causes overexpression of TNF-α and NO, which are known to depress myocardial contractile function.

Another possible mechanism is that calpain activation leads to degradation of IkB, which is an essential step in the translocation of transcription factor NF-κB from the cytosol to the nucleus. NF-κB then binds to the promoter region of genes encoding various inflammatory mediators or proteins such as TNF-α, interleukin (IL)-1, IL-6, vascular molecule-1, inducible NO synthase and cyclooxygenase 2.

Li et al used cultured adult cardiomyocytes and an in vivo model of endotoxia to investigate the role of calpain inhibitor, calpastatin, in lipopolysaccharide (LPS)-stimulated calpain activation and myocardial function. Their results suggest that overexpression of calpastatin inhibits caspase-3 activation and TNF-α expression in LPS-stimulated cardiomyocytes and improves cardiac function in endotoxia. Inhibition of calpain activity may improve myocardial function by the means of attenuation of tissue leukocyte infiltration and endothelial cell activation in the inflamed coronary vasculature. Tissier et al found that calpain inhibitors attenuate leukocyte endothelial in the mesenteric venules in a rat model of endotoxemia, which is consistent with previous studies showing that organ injury is attenuated by antileukocyte therapies. They also found that calpain inhibition improved myocardial contractile dysfunction in the same rat model. Calpain inhibition via its antiinflammatory effects may help to reduce atherosclerosis in diseased myocardium.

**Calpain Overactivation in Diabetes and Obesity**

Interestingly, calpain overactivation has been implicated in the pathogenesis of many of the comorbidities associated with CAD, including diabetes and obesity. Calpains have been found to play a role in the cardiac hypertrophy of diabetic cardiomyopathy. The inappropriate activation of high glucose-induced proteolytically cleaved calpain-1 promoted cellular apoptosis, scavenging of ROS, and chelation of excessive calcium. Inhibition of calpain-1 significantly reduced calpain activation and prevented cellular apoptosis during elevated glucose conditions. The proapoptotic role of high glucose-induced proteolytically cleaved calpain-1 was facilitated through activation of caspase-12 and caspase-9.

Endothelial calpain is known to be activated acutely by hyperglycemia. Insulin-dependent DM has been found to activate endothelial calpain through the protein kinase C signaling pathway.
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Calpain inhibition promoted expression of the survival proteins, decreased oxidative stress and inhibited apoptotic pathways in animals with MetS and chronic myocardial ischemia.\(^1,2\)

Conclusions

Excessive activation of calpains has been implicated in the pathophysiology of several disorders including CAD, myocardial IR injury, DM and obesity. Calpain overactivation is involved in number of cellular processes including cytoskeletal remodeling, cellular apoptosis, tissue angiogenesis, and endothelial inflammation. Calpain inhibition, therefore, may offer a novel potential medical therapy for patients suffering from a number of diseases, including CAD. Calpain inhibitors are experimental drugs and have not yet been developed as therapeutic agents in patients. Though the results of these studies are encouraging, further studies are merited to help elucidate the mechanism and extent by which calpain inhibitors exert their beneficial effects (Figure 3).

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References