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# Targeting calpains in myocardial ischemia/reperfusion injury

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Loss of intracellular Ca<sup>2+</sup> homeostasis occurring during ischemia/reperfusion (IR) induces dysregulated overactivation of calpains, Ca<sup>2+</sup>-dependent cysteine proteases. In this review we describe the mechanisms of calpain activation and analyze the evidence for calpain contribution to myocardial reperfusion injury. Exaggerated calpain activation occurs during reperfusion after intracellular pH normalization, and results in altered contractility and cell death through the cleavage of numerous protein substrates involved in sarcolemmal structure, cellular contractility, mitochondrial function, and cellular signalling. Finally, we provide an overview of the calpain inhibitors that have been evaluated in experimental models of IR and the advances in the design of new potent and selective compounds that may serve as candidates for testing the efficacy of calpain inhibition in patients with acute myocardial infarction.

### 1. Introduction

The pathophysiology of reperfusion injury is a complex process that causes cardiomyocyte death via multiple mechanisms and involves the alteration and interplay of a variety of molecules and proteins (Davidson et al., 2019). A pronounced consequence of reperfusion is the generation of intracellular Ca<sup>2+</sup> overload and a burst of reactive oxygen species (ROS), which have the potential to induce an anomalous increase in the activity of several enzymes. One of the families of proteins directly modulated by changes in the intracellular Ca<sup>2+</sup> concentration are calpains, Ca<sup>2+</sup>-dependent, non-lysosomal cysteine proteases (Goll et al., 2003). Calpains are essential for many physiological processes regulated by Ca<sup>2+</sup> including cell spreading and migration (Dewitt and Hallett, 2020), cell cycle (Santella, 1998), membrane repair (Mellgren et al., 2007), embryonic development (Arthur et al., 2000), and platelet function (Azam et al., 2001). In contrast to other major proteolytic systems, calpains do not induce protein digestion but act in a regulatory way by performing limited proteolysis of their substrates. Its activity is tightly controlled by their specific endogenous inhibitor calpastatin and the energy-dependent regulation of Ca<sup>2+</sup> homeostasis (Goll et al., 2003). However, under pathological conditions associated with the loss of intracellular Ca<sup>2+</sup> control, as occurs during ischemia/reperfusion

(IR), calpains are overactivated resulting in the proteolysis of a wide variety of proteins (Inserte et al., 2012). The use of transgenic models and calpain inhibitors demonstrate that calpain deregulation contributes to myocardial reperfusion injury through different mechanisms, which include increased sarcolemmal fragility and defects in Ca<sup>2+</sup> handling, altered myofibrillar contractility, and mitochondrial dysfunction. Calpains are sensitive to cellular acidosis and they appear to activate only during reperfusion, once intracellular pH has been normalized (Hernando et al., 2010). This feature is critical because it provides a window of opportunity for preventing their activation. Yet, confirmation of the potential cardioprotective value of strategies designed to prevent calpain activation in clinically relevant experimental models is scarce, mainly due to the lack of appropriate compounds. Development of potent and selective calpain inhibitors is challenging, but ongoing research in the pharmaceutical industry and academia continuously generates potential candidates based on new structures or in the optimization of current compounds (Ono et al., 2016). As a result of these efforts, some of these compounds have entered clinical trials to test their safety and pharmacodynamics (Lon et al., 2019) (jRTC2021190013). Finally, recent data demonstrating the involvement of calpains in the development of adverse postinfarction myocardial remodelling has re-

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awakened interest for calpains as potential treatment target in patients with acute myocardial infarction.

## 2. Regulation of the calpain system

At present, 15 calpain isoforms have been identified in humans. Some of these isoforms have tissue-specific distribution whereas others, including the most abundant and best studied forms, calpain-1 and calpain-2, are ubiquitously expressed. Both calpains are heterodimers composed of a large (80kDa) catalytic subunit (*Capn1* and *Capn2*, respectively), and a small (30kDa) common regulatory subunit (*Capn4*) needed to maintain stability and activity of the large subunit (Goll et al., 2003). These two isoforms differ in the concentration of  $Ca^{2+}$  required for their activation *in vitro* (Ono et al., 1998), which is substantially greater than the cytosolic concentrations achieved in normal beating cardiomyocytes. Additional mechanisms have been therefore proposed to contribute to lower the  $Ca^{2+}$  threshold of calpain activation reported *in vivo*. The most accepted one includes the translocation of calpain in its inactive form from the cytosol to the membrane where it binds to phospholipids reducing its  $Ca^{2+}$  requirements (Chakrabarti et al., 1996) or localizes in microdomains with high local  $Ca^{2+}$  concentrations (Kifor et al., 2003). However, although membranes are generally considered the preferential site of calpain activity, different studies suggest that under conditions of severe  $Ca^{2+}$  overload as those present during ischemia, translocation is not a critical step for calpain activation (Liu et al., 2001; Hernando et al., 2010). Calpastatin is the only specific endogenous inhibitor of calpain-1 and calpain-2. However, calpastatin has been shown to be largely downregulated during reperfusion due to the proteolytic activity of calpains, reducing its inhibitory function (Sorimachi et al., 1997; Hernando et al., 2010).

Several studies suggest that the oxidative/nitrosative stress generated during the first minutes of reperfusion may modify the activity of calpains. However, the mechanisms involved in the redox-regulation of calpain activity and their overall relevance in the context of acute reperfusion injury are still not fully understood (Randriamboavonjy et al., 2019). On the one hand, ROS can activate calpains indirectly via changes in  $Ca^{2+}$  levels. It has been described that ROS can elevate cytosolic  $Ca^{2+}$  by favouring calcium release from the sarcoplasmic reticulum and decreasing  $Ca^{2+}$  removal from the cell due to the oxidative damage of  $Ca^{2+}$  transporters (Itoh et al., 1999; Menshikova and Salama, 2000; Zima and Blatter, 2006). On the other hand, different studies suggest that calpain activity is inhibited by direct oxidation and S-nitrosylation of the protease (Michetti et al., 1995; Guttman and Johnson, 1998). The resulting inactivation of calpains could partially explain the proposed cardioprotective effects of nitrosative signalling against reperfusion injury (Penna et al., 2014; Totzeck et al., 2017). Other post-translational modifications that have been proposed to modulate calpain activity include phosphorylation by protein kinase A (PKA) and extracellular signal-regulated kinase (ERK) (Shiraha et al., 2002; Glading et al., 2004), and carbonylation (Norberg et al., 2010). Although PKA and ERK have been implicated in the mechanisms of IR injury and cardioprotection (Hausenloy and Yellon, 2006), and IR has been shown to increase protein nitrosylation (Hamilton et al., 2004) and carbonylation (Khaliulin et al., 2006), whether or not these protein modifications are relevant modulatory mechanisms of calpain activation during IR compared to  $Ca^{2+}$  overload has not yet been determined.

**Highlights:** calpain activity is tightly regulated by variations in  $Ca^{2+}$  concentration and calpastatin. Although post-translational modifications of calpains have been reported, their pathophysiological relevance remains to be determined.

## 3. Calpain contribution to acute myocardial reperfusion injury

Experimental evidence obtained using several independent approaches, including the cleavage of known calpain substrates, the measurement of cellular calpain activity, and the use of calpain inhibitors and transgenic animals with altered calpain system in a variety of experimental models of IR, unambiguously demonstrate that calpains are important contributors to IR injury in the heart (Inserte et al., 2012; Neuhof, 2014) (see Table 1). This is not surprising, as IR is associated with a significant and sustained alteration of  $Ca^{2+}$  homeostasis in cardiomyocytes (Garcia-Dorado et al., 2012). Importantly, intracellular pH (pHi) determines the time-course of calpain activation secondary to transient ischemia. Our group has reported that ischemia-induced intracellular acidosis inhibits calpain activation despite  $Ca^{2+}$  overload, while the rapid pHi normalization during reperfusion allows its overactivation (Hernando et al., 2010). In agreement with that, experimental interventions applied in Langendorff-perfused hearts specifically addressed to delay pHi recovery (i.e. acidic perfusion) or those interfering with pHi normalization during the first minutes of reperfusion (i.e. ischemic postconditioning), attenuated calpain activation and reduced reperfusion injury (Inserte et al., 2009). The causative role of calpains on reperfusion-induced cell death has been established by experiments in which the administration of calpain inhibitors exclusively during reperfusion reduced infarct size in *in vivo* and *in situ* models of IR (Neuhof et al., 2008; Hernando et al., 2010). Several mechanisms, summarized in Figure 1, have been proposed to explain the contribution of calpains to reperfusion injury.

### 3.1 Sarcolemmal fragility

Calpain activation during IR is implicated in the degradation of various sarcolemmal and cytoskeletal proteins (Neuhof, 2014). Calpain-dependent cleavage of  $\alpha$ -fodrin, which form the backbone of the membrane cytoskeleton (Bennett and Gilligan, 1993), has been associated with increased membrane fragility (Armstrong et al., 2001). Upon conditions of mechanical stress, as those imposed by myocardial reperfusion,  $\alpha$ -fodrin cleavage reduces the tolerance of the sarcolemma to the mechanical stress induced by cell contraction and swelling and increases the probability of sarcolemmal rupture and cardiomyocyte necrosis (Inserte et al., 2004). Other calpain substrates that may contribute to increase sarcolemmal fragility include dystrophin, talin, paxilin, vinculin, filamin, tau,  $\alpha$ -tubulin, and vimentin (Davies et al., 1978; Nelson and Traub, 1983; Yoshida et al., 1992; Franco et al., 2004; Cortesio et al., 2011).

### 3.2 Impairment of $Ca^{2+}$ homeostasis

Calpain degradation of  $\alpha$ -fodrin not only increases sarcolemmal fragility but in concert with the cleavage of ankyrin causes the detachment of several membrane receptors and channels including the  $\alpha$ -subunit of  $Na^+/K^+$  ATPase and the  $Na^+/Ca^{2+}$  exchanger from their anchorage to the ankyrin-fodrin complex, which is essential for their proper membrane localization and correct function (Li et al., 1993; Mohler et al., 2003). As a consequence, the elevated cytosolic  $Na^+$  concentration present in ischemic cells cannot be normalized upon reperfusion, resulting in further aggravation of  $Ca^{2+}$  overload through reduced  $Ca^{2+}$  extrusion, following a vicious circle that aggravates reperfusion injury (Inserte et al., 2005; Müller et al., 2013a). In addition, it has been suggested that calpains may impair sarcoplasmic reticulum control of cytosolic  $Ca^{2+}$  by cleaving sarcoplasmic reticulum  $Ca^{2+}$  ATPase (SERCA2) and the ryanodine receptor (Singh et al., 2004b; French et al., 2006; Pedrozo et al., 2010).

### 3.3 Myofibrillar derangement

Calpains have been largely involved in post-ischemic

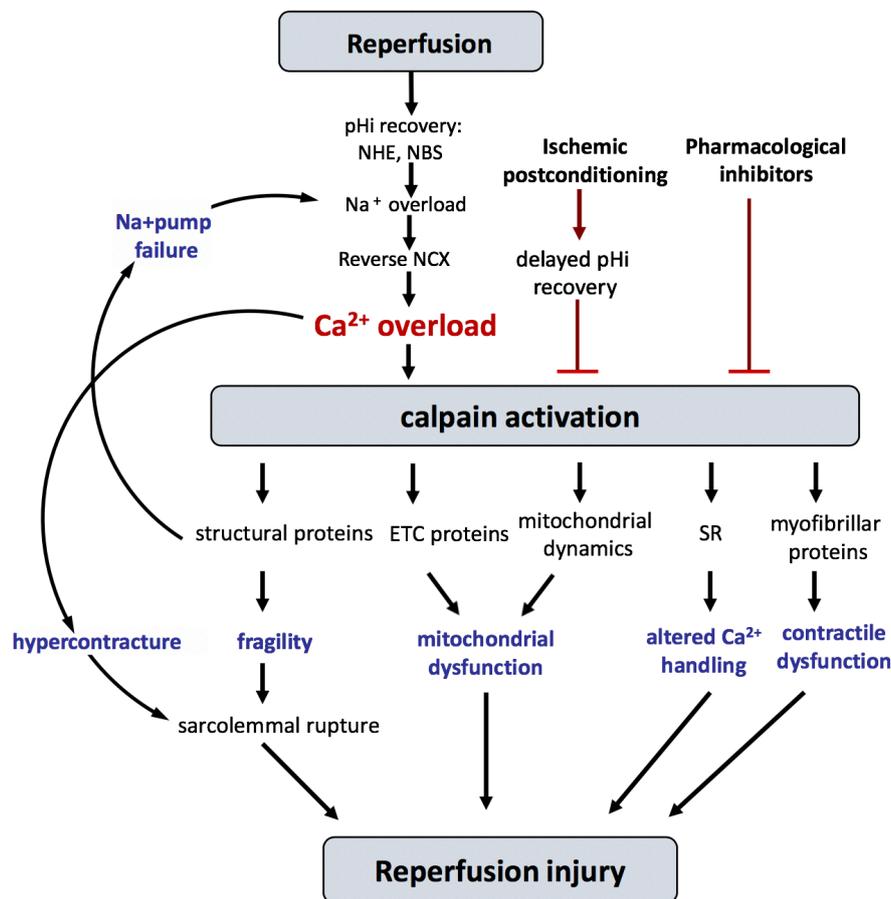


Figure 1. Schematic diagram showing the main proposed mechanisms discussed in the text by which calpains participate in reperfusion injury. ETC, electron transport chain; NCX,  $\text{Na}^+/\text{Ca}^{2+}$  exchanger; NBC,  $\text{Na}^+/\text{HCO}_3^-$  cotransporter; NHE,  $\text{Na}^+/\text{H}^+$  exchanger; pHi, intracellular pH; SR, sarcoplasmic reticulum.

myocardial dysfunction through the proteolysis of several proteins involved in cell contraction. The calpain dependent cleavage of titin, desmin, cardiac troponin T (cTnT), cardiac troponin I (cTnI) and  $\alpha$ -actinin is described in different experimental models of IR (Kositprapa et al., 2000; Maekawa et al., 2003; Zhang et al., 2006; Blunt et al., 2007; Feng et al., 2008; Li et al., 2009) and has been reported in human myocardial samples (Barta et al., 2005). Overexpression of calpastatin prevents the degradation of cTnI and attenuates contractile dysfunction in rat hearts subjected to IR (Maekawa et al., 2003). The regulation of its degradation by calpain and the identification of the cleavage sites is being exhaustively investigated (Wijnker et al., 2015; Li et al., 2017; Martin-Garrido et al., 2018; Mahmud et al., 2019). Overexpression of heat shock protein-27 (HSP27) has been proposed to improve post-ischemic contractility by preventing the proteolytic cleavage of cTnI, cTnT, and desmin via blockade of calpain interaction with these proteins (Blunt et al., 2007; Lu et al., 2008). Cardiac myosin binding protein-C (cMyBP-C) is hydrolyzed by calpain during IR generating a N-terminal fragment (Sadayappan et al., 2008) that it is also observed in infarcted human hearts (Barefield et al., 2019). Recently, it has been reported that the cardiac-specific ablation of the calpain-target site of cMyBP-C reduces myocardial infarction in an *in situ* IR model (Barefield et al., 2019). Junctophilin-2, an essential protein for efficient excitation-contraction coupling in adult cardiomyocytes, is reduced after transient ischemia leading to cardiac contractile dysfunction and heart failure due to the calpain-dependent cleavage of its N-terminal domain (Murphy et al., 2012; Guo et al., 2015). More recently, it has

been proposed that the resulting junctophilin-2 N-terminal fragment translocates to the nucleus where it acts as a stress-adaptive transcription regulator (Guo et al., 2018).

### 3.4 Mitochondrial dysfunction

Although traditionally considered cytoplasmic proteases, different studies have reported that calpains 1, 2, 4, and 10 are also present in the mitochondria (Arrington et al., 2006; Chen et al., 2011; Shintani-Ishida and Yoshida, 2015). Mitochondrial calpain-1 (mCPN1) is detected in the inter-membrane space (Ozaki et al., 2007) and matrix (Chen and Lesnefsky, 2015), and mitochondrial calpain-2 (mCPN2) in the matrix of mouse heart mitochondria (Shintani-Ishida and Yoshida, 2015). Studies performed in isolated liver mitochondria suggest that the activity of mCPN1 and mCPN2 is regulated through their association to the molecular chaperones endoplasmic reticulum resident protein 57 (ERp57) and glucose regulated protein 75 (Grp75), respectively (Ozaki et al., 2008, 2009). Calpain activation during IR were initially involved in the induction of the mitochondrial-dependent apoptotic programme by activating the pro-apoptotic factors BH3 interacting-domain death agonist (BID) (Chen et al., 2001; Luo et al., 2015) and apoptosis-inducing factor (AIF) (Chen et al., 2011). However, the relevance of apoptotic cardiomyocyte death in the context of acute reperfusion injury has been questioned due to the repression of the canonical caspase pathway in post-mitotic cardiomyocytes (Sanchis et al., 2008; Inserte et al., 2016). By contrast, several recent studies have proposed that mitochondrial calpains alter the electron transporter chain function by targeting the NADH dehydrogenase [ubiquinone]

iron-sulfur protein 7 (NDUFS7) (Chen et al., 2019) and NADH-ubiquinone oxidoreductase chain 6 protein (ND6) (Shintani-Ishida and Yoshida, 2015) subunits of complex I. Pharmacological inhibition of calpains in rats subjected to *ex vivo* and *in situ* transient ischemia prevented mitochondrial calpain activation, complex I cleavage and inactivation, and reduced the occurrence of mitochondria permeability transition pore (mPTP) (Shintani-Ishida and Yoshida, 2015; Thompson et al., 2016; Chen et al., 2019). Mitochondrial calpains have also been involved in the disruption of the mitochondrial F<sub>0</sub>F<sub>1</sub> ATP synthase through the proteolysis of its ATP5A1 subunit (Ni et al., 2016). Recently, by using transgenic mice with upregulation of calpain-1 restricted to cardiomyocyte mitochondria, the same group has proposed that calpain-mediated cleavage of ATP5A1 is causally associated with ROS generation, mPTP opening, and cell death (Cao et al., 2019).

Finally, mitochondrial calpains have been suggested to modulate mitochondrial dynamics. Recent findings suggest that impairment of mitochondrial dynamics may contribute to myocardial damage caused by IR (Ong et al., 2010; Forte et al., 2020). Cardiac-specific downregulation of OPA1 has been associated with mitochondrial fusion, mitophagy inhibition, and enhanced reperfusion injury (Zhang et al., 2019). In a recent study, calpastatin overexpression in mice subjected to *in vivo* transient ischemia prevented OPA1 degradation and mitochondrial fission, and improved mitochondrial fusion and mitophagy (Guan et al., 2019). Furthermore, calpain inhibition attenuated beclin-1 cleavage, a key component of the autophagy pathway required to form autophagosomes, and improved mitophagy in *ex vivo* hearts subjected to IR (Chen et al., 2019), suggesting that calpains negatively modulate mitophagy by acting at multiple levels.

### 3.5 Modulation of signaling pathways

Other known calpain substrates are protein kinases, protein phosphatases, and transcription factors (Neuhof, 2014). The calpain-dependent cleavage of these regulatory proteins may alter the function of several signaling pathways at different levels and may indirectly affect reperfusion injury. Proteolytic processing of protein kinase C (PKC) by calpain-1 generates a C-terminal fragment with unregulated kinase properties that contributes to reperfusion injury (Kang et al., 2010). Calpain activity also induces the activation of glycogen synthase kinase 3-beta (GSK-3 $\beta$ ) (a well-known regulator of mPTP opening) through the cleavage of its inhibitory domain, while its inhibition upregulates the GSK-3 $\beta$  downstream signaling pathways insulin/phosphoinositide-3-kinase (PI3K) and WNT/ $\beta$ -catenin (Potz et al., 2017). Different groups have demonstrated that calpain activation during reperfusion induces the cleavage of the nuclear factor kappa B (NF $\kappa$ B) inhibitor I $\kappa$ B $\alpha$ , inducing the translocation of NF $\kappa$ B to the nucleus, where it promotes the expression of proteins involved in the development of adverse ventricular remodeling (Hamid et al., 2011; Ma et al., 2012; Poncelas et al., 2017). In addition, it has been proposed that calpains elicit calcium-triggered cell injury by activating calcineurin in a calmodulin-independent form through the cleavage of its 60 kDa subunit (Lakshmikuttyamma et al., 2003) and the proteolysis of the calcineurin inhibitor cain/cabin1 (Kim et al., 2002). Finally, *in vitro* experiments show that purified m-calpain promotes partial degradation of G protein-coupled receptor kinase 2 (GRK2) and inhibits its activity (Lombardi et al., 2002). GRK2 is a central regulator of  $\beta$ -adrenergic receptors and many other G protein-coupled receptors (GPCR) involved in cardiovascular pathophysiology, but also a key regulatory node in non-GPCR pathways (Mayor et al., 2018). Recently we have reported that GRK2 levels are transiently reduced at the onset of reperfusion, at least in part due to the action of calpains, which reduced endogenous

cardioprotection through impaired overall protein kinase B (Akt) functionality (Penela et al., 2019).

**Highlights:** A broad range of experimental studies demonstrates that calpain activation contributes to IR injury by different mechanisms including increased sarcolemmal fragility, impairment of Ca<sup>2+</sup> regulation, myofibrillar derangement, mitochondrial dysfunction, and modulation of signaling pathways.

### 4. Pharmacological inhibition of calpains

Despite the compelling experimental evidence supporting the contribution of calpains to myocardial reperfusion injury, no clinical trials and only a few pre-clinical studies in large animal models have explored the use of pharmacological calpain inhibitors as a therapeutic strategy to attenuate myocardial infarction (see Table 1). This is in part due to the limitations of most of the available calpain inhibitors, which include poor selectivity (Ali et al., 2012), metabolic instability, limited membrane permeability, and poor water-solubility. The association of other non-cardiac pathological conditions, including myopathies, neurodegenerative disorders, ophthalmic diseases, or cancer with disturbances in the calpain system and the demonstration that transgenic models with deficient calpain expression or overexpression of calpastatin attenuate the symptoms of these pathologies (Zatz and Starling, 2005), make inhibition of calpains an attractive target for the industry, promoting active research for development of novel calpain inhibitors (examined in detail in previous reviews (Ono et al., 2016; Dókus et al., 2020)).

Leupeptin and E-64, both naturally isolated from microorganisms, constitute the first-generation of calpain inhibitors. These peptide aldehydes have an electrophilic center for a covalent interaction with the catalytic cysteine residue of calpain. Leupeptin was the first inhibitor tested in the context of IR injury, improving functional recovery in isolated guinea pig hearts subjected to IR (Matsumura et al., 1993). However, although these inhibitors have been extensively used for many years in *in vitro* and *in vivo* studies, they exhibit little specificity for calpains and low membrane permeability (Mehdi, 1991). The physicochemical properties of leupeptin were improved by substituting the terminal amino acid for a hydrophobic cap group (Donkor, 2011). The resulting synthetic leupeptin derivatives calpeptin, ALLN (calpain inhibitor I) and MDL-28170 (calpain inhibitor III) have demonstrated an improved efficacy to protect against reperfusion injury in isolated hearts and *in vivo* models of transient coronary occlusion (Inserte et al., 2004; Mani et al., 2009; Gilchrist et al., 2010; Hernando et al., 2010). Nevertheless, the high chemical reactivity of aldehydes and poor aqueous solubility limits the stability and bioavailability of these compounds, therefore precluding their progression into the clinic. Efforts to solve these problems have led to the development of peptidomimetic inhibitors derived from the MDL-28170 structure with improved water solubility, cell permeability, and metabolic stability over previous inhibitors (Lubisch et al., 2003). Among them, the benzoylalanine-derived ketoamides A-705239 and A-790253 developed by AbbVie reduced infarct size and mitochondrial dysfunction in isolated rabbit hearts (Neuhof et al., 2003, 2004) and in an *in vivo* pig model of IR (Khalil et al., 2005). An improved derivative of A-705239 namely Alicapistat (ABT-957), with high calpain selectivity versus cathepsins (Jantos et al., 2019), has been recently tested in a phase I clinical study analysing its safety and pharmacological properties for the treatment of Alzheimer's disease (Lon et al., 2019). However, in this study, Alicapistat failed to induce a measurable pharmacodynamic effect, which can be a consequence of selecting inadequate concentrations, suggesting that this

Table 1. Selected studies of calpain inhibition in ischemia/reperfusion models.

Inhibitor or TG	Species	Model	I/R Protocol	Effect	References
Leupeptin	Pig	Ex vivo	15'/20'	↑ LVDP, ↑ Maximal Ca <sup>2+</sup> -activated pressure (MCAP)	(Matsumura et al., 1993)
	Pig	Ex vivo	15'/20'	↑ LVDP, ↑ contractile function, ↓ damage of cytoskeletal proteins	(Zhang et al., 2012)
	Rat	Ex vivo	30'/60'	↑ contractile function, ↑ SR function	(Singh et al., 2004a)
	Rat	Ex vivo	30'/60'	↑ cardiac function, ↑ Na <sup>+</sup> /K <sup>+</sup> -ATPase levels and activity	(Singh and Dhalla, 2010)
E-64	Rat	Ex vivo	60'/30'	↑ α-fodrin, ↓ CK release	(Yoshida et al., 1995)
Calpeptin	Mice	In vivo	Permanent ischemia	↑ EF, ↓ EDV, ↓ cell death	(Mani et al., 2009)
	Isolated cardiomyocytes	Ex vivo	20'/30'	↑ cell shortening, ↑ Ca <sup>2+</sup> transients, ↓ Ca <sup>2+</sup> overload	(Zhang et al., 2012)
ALLN (Calpain inhibitor-1)	Rat	Ex vivo	10-60'/30'	↓ degradation of caldesmon, ↓ myocardial stunning	(Yoshida et al., 1995)
	Pig	Ex vivo	15'/20'	↑ LVDP, ↑ contractile function, ↓ damage of cytoskeletal proteins	(Zhang et al., 2012)
	Rat	In vivo	30'/6h	↓ infarct size	(Iwamoto et al., 1999)
	Rat	Ex vivo	15'/60'	↑ End-systolic pressure, ↑ Systolic pressure-volume area, ↑ oxygen consumption, ↑ α-fodrin	(Yoshikawa et al., 2005)
	Rat	Ex vivo	27'/60'	↓ calpain autolysis, ↑ α-fodrin, Earlier recovery in LVDP	(Gilchrist et al., 2010)
	Rabbit	Ex vivo	30'/15-90'	↓ cleavage of Bid, ↓ CK release, ↓ Infarct size	(Chen et al., 2012)
	Rat	In vivo	30'/30'	↓ Infarct size, ↓ mPTP opening, ↓ ND truncation, ↓ complex I inactivation	(Shintani-Ishida and Yoshida, 2015)
MDL-28170	Rat	Ex vivo	60'/30'	↑ α-fodrin, ↑ cardiac function, ↓ LDH release	(Inserre et al., 2004)
	Rat	Ex vivo	60'/30'	↑ α-fodrin and ankyrin-B, ↑ Na <sup>+</sup> /K <sup>+</sup> -ATPase activity, ↓ LDH release, ↑ contractile recovery	(Inserre et al., 2005)
	Rat	Ex vivo	60'/30'	↑ cardiac function, ↓ LDH release, ↓ infarct size	(Inserre et al., 2006)
	Rat	Ex vivo	25'/45'	↑ cardiac work, ↓ LDH release, ↑ spectrin, talin, calpastatin	(French et al., 2006)
	Rat	In vivo	30'/120'	↓ infarct size	(Hernando et al., 2010)
	Mice	Ex vivo	30'/30'	↓ AIF cleavage	(Chen et al., 2011)
	Rat	Ex vivo	30'/30'	↑ cardiac function, ↑ Na <sup>+</sup> /K <sup>+</sup> -ATPase activity	(Singh and Dhalla, 2010)
	Rat	Ex vivo	30'/30'	↓ MMP activity, ↑ cardiac function ↑ Na <sup>+</sup> /K <sup>+</sup> -ATPase activity	(Singh et al., 2012)
	Mouse	Ex vivo	25'/30'	↑ calcium retention capacity, ↓ mPTP opening	(Singh et al., 2014)
	Mice	Ex vivo	35'/50'	↓ JP2 downregulation	(Guo et al., 2015)
	Mice	Ex vivo	25'/30'	↓ LDH release, ↓ spectrin cleavage ↓ mPTP opening, protected complex I activity	(Thompson et al., 2016)
A-705239 (BSF 409425)	Rabbit	Ex vivo	30 or 45'/60'	↑ state 3 respiration, ↓ state 4 respiration, ↓ mitochondria leaks	(Trumbeckaite et al., 2003)
A-705239 (BSF 409425)/A-705253	Rabbit	Ex vivo	60'/120'	↓ infarct size	(Neuhof et al., 2004)
A-705253	Pig	In vivo	45'/6h	↓ infarct size, ↑ left ventricular contractility, ↑ global hemodynamic function	(Khalil et al., 2005)
A-705253	Rabbit	Ex vivo	60'/120'	↓ infarct size	(Neuhof et al., 2008)
SNJ-1945	Rat	Ex vivo	30'*/60'	↑ α-fodrin, ↓ cardiac dysfunction	(Yoshikawa et al., 2010)
	Rat	Ex vivo	30'/Different times	↑ LTCC and SERCA2a, ↓ cardiac dysfunction	(Takeshita et al., 2013)
	Rat	In vivo	30'-90' /Different times	↓ myoglobin level	(Sonobe et al., 2014)
	Rat	In vivo	30'/24h	↓ infarct size	(Poncelas et al., 2017)
	Rat	Ex vivo	40'/Different times	↓ infarct size, ↑ GRK2, Pin1 and AKT levels	(Penela et al., 2019)
PD150606	Mice	In vivo	30'/24h	↓ infarct size, ↓ apoptosis, ↓ mitochondrial dysfunction	(Luo et al., 2015)
Calpastatin overexpression	Rat	Ex vivo	30'/60'	↓ contractile dysfunction, ↓ troponin I degradation	(Maekawa et al., 2003)
	Mice	Ex vivo	45'/30'	↑ cardiac function, ↓ LDH release	(Shan et al., 2010)
	Mice	In vivo	Permanent ischemia	↓ infarct size	(Ye et al., 2015)
	Mice	Ex vivo	35'/50'	↓ JP2 downregulation	(Guo et al., 2015)
	Mice	In vivo	30'/120'	↑ mitochondrial fusion and mitophagy, ↓ mitochondrion fission and apoptosis	(Guan et al., 2019)
Calpain1 knockout	Mice	Ex vivo	40'/120'	↓ infarct size	(Kang et al., 2010)

CK: creatine kinase; EDV: end-diastolic ventricular volume; EF: ejection fraction; LDH: lactate dehydrogenase; LTCC: L-type calcium channel; LVDP:left ventricular developed pressure; MMP: metalloproteinase; ND: NADH-ubiquinone oxidoreductase chain 6 protein; SR: sarcoplasmic reticulum; TG: transgenic model

inhibitor has indeed a moderate potency. Another ketoamide derived inhibitor with an encouraging pharmacokinetic profile is SNJ-1945, produced by Senju Pharmaceutical (Shirasaki et al., 2006; Yoshikawa et al., 2010). Intraperitoneal administration of SNJ-1945 attenuated ventricular dysfunction induced by IR (Takeshita et al., 2013) and reduced infarct size when given immediately before reperfusion in *in vivo* rat and mouse models of transient coronary occlusion (Poncelas et al., 2017; Penela et al., 2019). Currently, a phase II clinical trial designed to test the efficacy and safety of oral administration of SNJ-1945 in central retinal artery occlusion is in progress (jRTC2021190013). The limited specificity for calpains over other cysteine proteases present in many calpain inhibitors has been attributed to the highly conserved active site among cysteine proteases. Therefore, a novel approach to develop new inhibitors with increased specificity for calpains is based on the use of compounds that induce allosteric inhibition of the enzyme by binding to other position than the catalytic site. One of these allosteric inhibitors, PD150606, which is supposed to bind to the Ca<sup>2+</sup> binding site of calpain, has been demonstrated to be effective in attenuating myocardial infarction in mice subjected to *in vivo* IR (Luo et al., 2015). Importantly, a derivative of PD150606 (PD151746) has been shown to be more selective for calpain-1 than for calpain-2. Because the two isoforms may display some differences in their substrate specificity (Shinkai-Ouchi et al., 2016) and biological function (Santos et al., 2012; Wang et al., 2016), this type of compounds opens the door to the development of new isoform-selective inhibitors. In addition to calpain isoforms, differences also seem to exist between cytosolic and mitochondrial calpains. It has been proposed that mitochondrial but not cytosolic calpains bind to chaperons (ERp57 for calpain-1 and Grp75 for calpain-2) (Ozaki et al., 2011). Based on these observations, Ozaki et al. (2012) developed a novel peptide that blocks the binding site between calpain-1 and ERp57 and inhibits the mitochondrial activity of calpain-1 in a specific manner. Considering the central role of mitochondria as a trigger of cell death during IR injury (Davidson et al., 2020), and that mitochondrial calpain-1 activity is increased in mice subjected to IR (Chen et al., 2011), the potential cardioprotective effects of these types of inhibitors deserve further investigation.

**Highlights:** Translation of the use of calpain inhibitors into clinical trials has been hampered due to the lack of inhibitors with appropriate pharmacological properties for use in patients. The development of new calpain inhibitors is an increasing research trend.

## 5. Perspectives

Calcium overload occurring during IR invariably results in aberrant activation of calpains and the concomitant cleavage of a wide variety of proteins. Calpain activation contributes to the aggravation of reperfusion injury by reducing the likelihood of a cell to survive. Several experimental studies have demonstrated in different models of IR that calpains are an attractive therapeutic target and that pharmacological inhibitors applied at the onset of reperfusion are capable of preventing cell death and limiting myocardial infarction. However, translation of the use of calpain inhibitors into clinical context has been frustrating, in part due to the lack of specific compounds with an appropriate pharmacologic profile to make them candidates for use in patients, but also due to the negative results obtained when using some promising cardioprotective interventions, like ischemic postconditioning (Engström et al., 2017), which has been shown to prevent calpain activity in experimental studies (Inserte et al., 2009), in patients with acute coronary syndrome.

Fortunately, due to the involvement of calpain in other non-cardiac pathologies, there is a high interest for the development of novel and specific calpain inhibitors, and plenty of molecules are being described in the literature and registered in patent offices. The safety and pharmacokinetics of some of the drugs that have demonstrated efficacy in animal models of IR are currently being tested for safety in clinical trials (jRTC2021190013; (Lon et al., 2019)). The 3D structure of the calpastatin-calpain complex has already been solved (Hanna et al., 2008; Moldoveanu et al., 2008) and it is being used to find new chemical structures that can be candidates for the design of new calpain inhibitors or for the optimization of the current compounds. One of the historical limitations of many calpain inhibitors is their poor selectivity over the cysteine proteases cathepsins (Mehdi, 1991). However, given the evidence for the involvement of the altered activity of both cathepsins and calpains in different pathophysiological conditions including myocardial infarction (Müller et al., 2013b), it has been questioned whether the design of pure specific inhibitors is essential for their clinical efficacy (Siklos et al., 2015).

In addition to their role in reperfusion injury, different studies using transgenic models have demonstrated that calpains participate in the progression of adverse post-infarction remodeling (Ma et al., 2012; Kudo-Sakamoto et al., 2014; Ye et al., 2015). In two recent studies, the chronic administration of SNJ1945 and MDL-28170 during reperfusion was effective in attenuating ventricular remodeling and cardiac dysfunction in a mouse model of transient coronary occlusion with no signs of toxic effects (Poncelas et al., 2017; Wang et al., 2018). Overall, these promising experimental results demonstrate that calpain inhibition is feasible and safe and may be an effective therapeutic intervention to attenuate both reperfusion injury and the development of adverse postinfarction remodeling and heart failure in patients with acute myocardial infarction.

**Highlights:** Recent studies suggest that calpains participate in the progression of post-infarction remodeling. However, translational research involving calpains is still at the development stage.

## Conflicts of interest:

The authors declare that they have no conflicts of interest.

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## References

- Ali MAM, Stepanko A, Fan X, Holt A, Schulz R (2012) Calpain inhibitors exhibit matrix metalloproteinase-2 inhibitory activity. *Biochem Biophys Res Commun* 423:1–5 Available at: <http://dx.doi.org/10.1016/j.bbrc.2012.05.005>.
- Armstrong SC, Latham CA, Shivel CL, Ganote CE (2001) Ischemic loss of sarcolemmal dystrophin and spectrin: Correlation with myocardial injury. *J Mol Cell Cardiol* 33:1165–1179.
- Arrington DD, Van Vleet TR, Schnellmann RG (2006) Calpain 10: A mitochondrial calpain and its role in calcium-induced mitochondrial dysfunction. *Am J Physiol - Cell Physiol* 291:1159–1171.
- Arthur JSC, Elce JS, Hegadorn C, Williams K, Greer PA (2000) 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* 20:4474–4481.
- Azam M, Andrabi SS, Sahr KE, Kamath L, Kuliopulos A,

- Chishti AH (2001) Disruption of the Mouse  $\mu$ -Calpain Gene Reveals an Essential Role in Platelet Function. *Mol Cell Biol* 21:2213–2220.
- Barefield DY et al. (2019) Ablation of the calpain-targeted site in cardiac myosin binding protein-C is cardioprotective during ischemia-reperfusion injury. *J Mol Cell Cardiol* 129:236–246.
- Barta J, Tóth A, Édes I, Vaszily M, Papp JG, Varró A, Papp Z (2005) Calpain-1-sensitive myofibrillar proteins of the human myocardium. *Mol Cell Biochem* 278:1–8.
- Bennett V, Gilligan DM (1993) The spectrin-based membrane skeleton and micron-scale organization of the plasma membrane. *Annu Rev Cell Biol*:27–66.
- Blunt BC, Creek AT, Henderson DAC, Hofmann PA (2007) H<sub>2</sub>O<sub>2</sub> activation of HSP25/27 protects desmin from calpain proteolysis in rat ventricular myocytes. *Am J Physiol - Hear Circ Physiol* 293.
- Cao T, Fan S, Zheng D, Wang G, Yu Y, Chen R, Song LS, Fan GC, Zhang Z, Peng T (2019) Increased calpain-1 in mitochondria induces dilated heart failure in mice: role of mitochondrial superoxide anion. *Basic Res Cardiol* 114:1–15.
- Chakrabarti AK, Dasgupta S, Gadsden RH, Hogan EL, Banik NL (1996) Regulation of brain m calpain Ca<sup>2+</sup> sensitivity by mixtures of membrane lipids: Activation at intracellular Ca<sup>2+</sup> Level. *J Neurosci Res* 44:374–380.
- Chen M, He H, Zhan S, Krajewski S, Reed JC, Gottlieb RA (2001) Bid is Cleaved by Calpain to an Active Fragment in Vitro and during Myocardial Ischemia/Reperfusion. *J Biol Chem* 276:30724–30728.
- Chen MC, Huang CY, Hsu SL, Lin E, Ku C Te, Lin H, Chen CM (2012) Retinoic acid induces apoptosis of prostate cancer DU145 cells through Cdk5 overactivation. *Evidence-based Complement Altern Med* 2012.
- Chen Q, Lesnefsky EJ (2015) Heart mitochondria and calpain 1: Location, function, and targets. *Biochim Biophys Acta - Mol Basis Dis* 1852:2372–2378.
- Chen Q, Paillard M, Gomez L, Ross T, Hu Y, Xu A, Lesnefsky EJ (2011) Activation of mitochondrial  $\mu$ -calpain increases AIF cleavage in cardiac mitochondria during ischemia-reperfusion. *Biochem Biophys Res Commun* 415:533–538.
- Chen Q, Thompson J, Hu Y, Dean J, Lesnefsky EJ (2019) Inhibition of the ubiquitous calpains protects complex i activity and enables improved mitophagy in the heart following ischemia-reperfusion. *Am J Physiol - Cell Physiol* 317:C910–C921.
- Cortasio CL, Boateng LR, Piazza TM, Bennin DA, Huttenlocher A (2011) Calpain-mediated proteolysis of paxillin negatively regulates focal adhesion dynamics and cell migration. *J Biol Chem* 286:9998–10006.
- Davidson SM, Adameová A, Barile L, Cabrera-Fuentes HA, Lazou A, Pagliaro P, Stenslökken KO, Garcia-Dorado D (2020) Mitochondrial and mitochondrial-independent pathways of myocardial cell death during ischaemia and reperfusion injury. *J Cell Mol Med* 24:3795–3806.
- Davidson SM, Ferdinandy P, Andreadou I, Bøtker HE, Heusch G, Ibáñez B, Ovize M, Schulz R, Yellon DM, Hausenloy DJ, Garcia-Dorado D (2019) Multitarget Strategies to Reduce Myocardial Ischemia/Reperfusion Injury: JACC Review Topic of the Week. *J Am Coll Cardiol* 73:89–99.
- Davies PJ, Wallach D, Willingham MC, Pastan I, Yamaguchi M, Robson RM (1978) Filamin-actin Interaction. Dissociation of Binding From Gelation by Ca<sup>2+</sup>-activated Proteolysis. *J Biol Chem* 253:4036–4042.
- Dewitt S, Hallett MB (2020) Calpain Activation by Ca<sup>2+</sup> and Its Role in Phagocytosis. *Adv Exp Med Biol* 1246:129–151.
- Dókus LE, Yousef M, Bánóczy Z (2020) Modulators of calpain activity: inhibitors and activators as potential drugs. *Expert Opin Drug Discov* 15:471–486.
- Donkor IO (2011) Calpain inhibitors: A survey of compounds reported in the patent and scientific literature. *Expert Opin Ther Pat* 21:601–636.
- Engström T et al. (2017) Effect of ischemic postconditioning during primary percutaneous coronary intervention for patients with ST-segment elevation myocardial infarction: A randomized clinical trial. *JAMA Cardiol* 2:490–497.
- Feng HZ, Biesiadecki BJ, Yu Z Bin, Hossain MM, Jin JP (2008) Restricted N-terminal truncation of cardiac troponin T: A novel mechanism for functional adaptation to energetic crisis. *J Physiol* 586:3537–3550.
- Forte M, Schirone L, Ameri P, Basso C, Catalucci D, Modica J, Chimenti C, Crotti L, Frati G, Rubattu S, Schiattarella GG, Torella D, Perrino C, Indolfi C, Sciarretta S (2020) The role of mitochondrial dynamics in cardiovascular diseases. *Br J Pharmacol*:0–1.
- Franco SJ, Rodgers MA, Perrin BJ, Han J, Bennin DA, Critchley DR, Huttenlocher A (2004) Calpain-mediated proteolysis of talin regulates adhesion dynamics. *Nat Cell Biol* 6:977–983.
- French JP, Quindry JC, Falk DJ, Staib JL, Lee Y, Wang KKW, Powers SK (2006) Ischemia-reperfusion-induced calpain activation and SERCA2a degradation are attenuated by exercise training and calpain inhibition. *Am J Physiol - Hear Circ Physiol* 290:128–137.
- Garcia-Dorado D, Ruiz-Meana M, Inserte J, Rodriguez-Sinovas A, Piper HM (2012) Calcium-mediated cell death during myocardial reperfusion. *Cardiovasc Res* 94.
- Gilchrist JSC, Cook T, Abrenica B, Rashidkhani B, Pierce GN (2010) Extensive autolytic fragmentation of membranous versus cytosolic calpain following myocardial ischemia-reperfusion. *Can J Physiol Pharmacol* 88:584–594.
- Glading A, Bodnar RJ, Reynolds IJ, Shiraha H, Satish L, Potter DA, Blair HC, Wells A (2004) Epidermal Growth Factor Activates m-Calpain (Calpain II), at Least in Part, by Extracellular Signal-Regulated Kinase-Mediated Phosphorylation. *Mol Cell Biol* 24:2499–2512.
- Goll DE, Thompson VF, Li H, Wei W, Cong J (2003) The calpain system. *Physiol Rev* 83:731–801.
- Guan L, Che Z, Meng X, Yu Y, Li M, Yu Z, Shi H, Yang D, Yu M (2019) MCU Up-regulation contributes to myocardial ischemia-reperfusion Injury through calpain/OPA-1-mediated mitochondrial fusion/mitophagy Inhibition. *J Cell Mol Med* 23:7830–7843.
- Guo A et al. (2018) E-C coupling structural protein junctophilin-2 encodes a stress-adaptive transcription regulator. *Science (80- )* 362:1–14.
- Guo A, Hall D, Zhang C, Peng T, Miller JD, Kutschke W, Grueter CE, Johnson FL, Lin RZ, Song LS (2015) Molecular determinants of calpain-dependent cleavage of junctophilin-2 protein in cardiomyocytes. *J Biol Chem* 290:17946–17955.
- Guttmann RP, Johnson GVW (1998) Oxidative stress inhibits calpain activity in situ. *J Biol Chem* 273:13331–13338.
- Hamid T, Guo SZ, Kingery JR, Xiang X, Dawn B, Prabhu SD (2011) Cardiomyocyte NF- $\kappa$ B p65 promotes adverse remodelling, apoptosis, and endoplasmic reticulum stress in heart failure. *Cardiovasc Res* 89:129–138.
- Hamilton KL, Quindry JC, French JP, Staib J, Hughes J, Mehta JL, Powers SK (2004) MnSOD antisense treatment and exercise-induced protection against arrhythmias. *Free Radic Biol Med* 37:1360–1368.
- Hanna RA, Campbell RL, Davies PL (2008) Calcium-bound structure of calpain and its mechanism of inhibition by calpastatin. *Nature* 456:409–412.

- Hausenloy DJ, Yellon DM (2006) Survival kinases in ischemic preconditioning and postconditioning. *Cardiovasc Res* 70:240–253.
- Hernando V, Insete J, Sartório CL, Parra VM, Poncelas-Nozal M, Garcia-Dorado D (2010) Calpain translocation and activation as pharmacological targets during myocardial ischemia/reperfusion. *J Mol Cell Cardiol* 49:271–279 Available at: <http://dx.doi.org/10.1016/j.yjmcc.2010.02.024>.
- Insete J, Barba I, Hernando V, Garcia-Dorado D (2009) Delayed recovery of intracellular acidosis during reperfusion prevents calpain activation and determines protection in postconditioned myocardium. *Cardiovasc Res* 81:116–122.
- Insete J, Cardona M, Poncelas-Nozal M, Hernando V, Vilarrosa Ú, Aluja D, Parra VM, Sanchis D, Garcia-Dorado D (2016) Studies on the role of apoptosis after transient myocardial ischemia: genetic deletion of the executioner caspases-3 and -7 does not limit infarct size and ventricular remodeling. *Basic Res Cardiol* 111:1–10.
- Insete J, Garcia-Dorado D, Hernando V, Barba I, Soler-Soler J (2006) Ischemic preconditioning prevents calpain-mediated impairment of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity during early reperfusion. *Cardiovasc Res* 70:364–373.
- Insete J, Garcia-Dorado D, Hernando V, Soler-Soler J (2005) Calpain-mediated impairment of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity during early reperfusion contributes to cell death after myocardial ischemia. *Circ Res* 97:465–473.
- Insete J, Garcia-Dorado D, Ruiz-Meana M, Agulló L, Pina P, Soler-Soler J (2004) Ischemic preconditioning attenuates calpain-mediated degradation of structural proteins through a protein kinase A-dependent mechanism. *Cardiovasc Res* 64:105–114.
- Insete J, Hernando V, Garcia-Dorado D (2012) Contribution of calpains to myocardial ischaemia/reperfusion injury. *Cardiovasc Res* 96:23–31.
- Itoh S, Yanagishita T, Aoki S, Koba S, Iwata T, Ishioka H, Arata H, Mukae S, Geshi E, Konno N, Katagiri T, Utsumi H (1999) Generation of free radicals and the damage done to the sarcoplasmic reticulum during reperfusion injury following brief ischemia in the canine heart. *Jpn Circ J* 63:373–378.
- Iwamoto H, Miura T, Okamura T, Shirakawa K, Iwatate M, Kawamura S, Tatsuno H, Ikeda Y, Matsuzaki M (1999) Calpain inhibitor-1 reduces infarct size and DNA fragmentation of myocardium in ischemic/reperfused rat heart. *J Cardiovasc Pharmacol* 33:580–586.
- Jantos K, Kling A, Mack H, Hornberger W, Moeller A, Nimmrich V, Lao Y, Nijssen M (2019) Discovery of ABT-957: 1-Benzyl-5-oxopyrrolidine-2-carboxamides as selective calpain inhibitors with enhanced metabolic stability. *Bioorganic Med Chem Lett* 29:1968–1973 Available at: <https://doi.org/10.1016/j.bmcl.2019.05.034>.
- Kang MY, Zhang Y, Matkovich SJ, Diwan A, Chishti AH, Dorn GW (2010) Receptor-independent cardiac protein kinase Ca activation by calpain-mediated truncation of regulatory domains. *Circ Res* 107:903–912.
- Khalil PN, Neuhof C, Huss R, Pollhammer M, Khalil MN, Neuhof H, Fritz H, Siebeck M (2005) Calpain inhibition reduces infarct size and improves global hemodynamics and left ventricular contractility in a porcine myocardial ischemia/reperfusion model. *Eur J Pharmacol* 528:124–131.
- Khaliulin I, Schneider A, Houminer E, Borman JB, Schwab H (2006) Apomorphine-induced myocardial protection is due to antioxidant and not adrenergic/dopaminergic effects. *Free Radic Biol Med* 40:1713–1720.
- Kifor O, Kifor I, Moore FD, Butters RR, Brown EM (2003) m-calpain colocalizes with the calcium-sensing receptor (CaR) in caveolae in parathyroid cells and participates in degradation of the CaR. *J Biol Chem* 278:31167–31176.
- Kim MJ, Jo DG, Hong GS, Kim BJ, Lai M, Cho DH, Kim KW, Bandyopadhyay A, Hong YM, Kim DH, Cho C, Liu JO, Snyder SH, Jung YK (2002) Calpain-dependent cleavage of cain/cabin1 activates calcineurin to mediate calcium-triggered cell death. *Proc Natl Acad Sci U S A* 99:9870–9875.
- Kositprapa C, Zhang B, Berger S, Canty JM, Lee TC (2000) Calpain-mediated proteolytic cleavage of troponin I induced by hypoxia or metabolic inhibition in cultured neonatal cardiomyocytes. *Mol Cell Biochem* 214:47–55.
- Kudo-Sakamoto Y, Akazawa H, Ito K, Takano J, Yano M, Yabumoto C, Naito AT, Oka T, Lee JK, Sakata Y, Suzuki JI, Saïdo TC, Komuro I (2014) Calpain-dependent cleavage of N-cadherin is involved in the progression of post-myocardial infarction remodeling. *J Biol Chem* 289:19408–19419.
- Lakshmikuttyamma A, Selvakumar P, Kakkar R, Kanthan R, Wang R, Sharma RK (2003) Activation of Calcineurin Expression in Ischemia-Reperfused Rat Heart and in Human Ischemic Myocardium. *J Cell Biochem* 90:987–997.
- Li X, Li Y, Shan L, Shen E, Chen R, Peng T (2009) Overexpression of calpastatin inhibits calpain activation and attenuates myocardial dysfunction during endotoxaemia. *Cardiovasc Res* 83:72–79.
- Li Y, Zhu G, Paolucci N, Zhang P, Takahashi C, Okumus N, Heravi A, Keceli G, Ramirez-Correa G, Kass DA, Murphy AM (2017) Heart failure-related hyperphosphorylation in the cardiac troponin I C terminus has divergent effects on cardiac function in vivo. *Circ Hear Fail* 10.
- Li Z, Burke EP, Frank JS, Bennett V, Philipson KD (1993) The cardiac Na<sup>+</sup>-Ca<sup>2+</sup> exchanger binds to the cytoskeletal protein ankyrin. *J Biol Chem* 268:11489–11491.
- Liu X, Rainey JJ, Harriman JF, Schnellmann RG (2001) Calpains mediate acute renal cell death: Role of autolysis and translocation. *Am J Physiol - Ren Physiol* 281:728–738.
- Lombardi MS, Kavelaars A, Penela P, Scholtens EJ, Roccio M, Schmidt RE, Schedlowski M, Mayor F, Heijnen CJ (2002) Oxidative stress decreases G protein-coupled receptor kinase 2 in lymphocytes via a calpain-dependent mechanism. *Mol Pharmacol* 62:379–388.
- Lon HK, Mendonca N, Goss S, Othman AA, Locke C, Jin Z, Rendenbach-Mueller B (2019) Pharmacokinetics, Safety, Tolerability, and Pharmacodynamics of Alicapostat, a Selective Inhibitor of Human Calpains 1 and 2 for the Treatment of Alzheimer Disease: An Overview of Phase 1 Studies. *Clin Pharmacol Drug Dev* 8:290–303.
- Lu XY, Chen L, Cai XL, Yang HT (2008) Overexpression of heat shock protein 27 protects against ischaemia/reperfusion-induced cardiac dysfunction via stabilization of troponin I and T. *Cardiovasc Res* 79:500–508.
- Lubisch W, Beckenbach E, Bopp S, Hofmann HP, Kartal A, Kästel C, Lindner T, Metz-Garrecht M, Reeb J, Regner F, Vierling M, Möller A (2003) Benzoylalanine-derived ketoamides carrying vinylbenzyl amino residues: Discovery of potent water-soluble calpain inhibitors with oral bioavailability. *J Med Chem* 46:2404–2412.
- Luo T, Yue R, Hu H, Zhou Z, Yiu KH, Zhang S, Xu L, Li K, Yu Z (2015) PD150606 protects against ischemia/reperfusion injury by preventing  $\mu$ -calpain-induced mitochondrial apoptosis. *Arch Biochem Biophys* 586:1–9.
- Ma J, Wei M, Wang Q, Li J, Wang H, Liu W, Laceyfield JC, Greer PA, Karmazyn M, Fan GC, Peng T (2012) Deficiency of Capn4 gene inhibits nuclear factor- $\kappa$ B

- (NF- $\kappa$ B) protein signaling/inflammation and reduces remodeling after myocardial infarction. *J Biol Chem* 287:27480–27489.
- Maekawa A, Lee JK, Nagaya T, Kamiya K, Yasui K, Horiba M, Miwa K, Uzzaman M, Maki M, Ueda Y, Kodama I (2003) Overexpression of calpastatin by gene transfer prevents troponin I degradation and ameliorates contractile dysfunction in rat hearts subjected to ischemia/reperfusion. *J Mol Cell Cardiol* 35:1277–1284.
- Mahmud Z, Zahran S, Liu PB, Reiz B, Chan BYH, Roczkowsky A, McCartney CSE, Davies PL, Li L, Schulz R, Hwang PM (2019) Structure and proteolytic susceptibility of the inhibitory C-terminal tail of cardiac troponin I. *Biochim Biophys Acta - Gen Subj* 1863:661–671.
- Mani SK, Balasubramanian S, Zavadzka JA, Jeffords LB, Rivers WT, Zile MR, Mukherjee R, Spinale FG, Kuppaswamy D (2009) Calpain inhibition preserves myocardial structure and function following myocardial infarction. *Am J Physiol - Hear Circ Physiol* 297:1744–1751.
- Martin-Garrido A, Biesiadecki BJ, Salhi HE, Shaifta Y, Dos Remedios CG, Ayaz-Guner S, Cai W, Ge Y, Avkiran M, Kentish JC (2018) Monophosphorylation of cardiac troponin-I at Ser-23/24 is sufficient to regulate cardiac myofibrillar Ca<sup>2+</sup> sensitivity and calpain-induced proteolysis. *J Biol Chem* 293:8588–8599.
- Matsumura Y, Kusuoka H, Inoue M, Hori M, Kamada T (1993) Protective effect of the protease inhibitor leupeptin against myocardial stunning. *J Cardiovasc Pharmacol* 22:135–142.
- Mayor F, Cruces-Sande M, Arcones AC, Vila-Bedmar R, Briones AM, Salices M, Murga C (2018) G protein-coupled receptor kinase 2 (GRK2) as an integrative signalling node in the regulation of cardiovascular function and metabolic homeostasis. *Cell Signal* 41:25–32.
- Mehdi S (1991) Cell-penetrating inhibitors of calpain. *Trends Biochem Sci* 16:150–153.
- Mellgren RL, Zhang W, Miyake K, McNeil PL (2007) Calpain is required for the rapid, calcium-dependent repair of wounded plasma membrane. *J Biol Chem* 282:2567–2575.
- Menshikova E V., Salama G (2000) Cardiac ischemia oxidizes regulatory thiols on ryanodine receptors: Captopril acts as a reducing agent to improve CA<sup>2+</sup> uptake by ischemic sarcoplasmic reticulum. *J Cardiovasc Pharmacol* 36:656–668.
- Michetti M, Salamino F, Melloni E, Pontremoli S (1995) Reversible inactivation of calpain isoforms by nitric oxide. *Biochem Biophys Res Commun* 207:1009–1014.
- Mohler PJ, Schott JJ, Gramolini AO, Dilly KW, Guatimosim S, DuBell WH, Song LS, Haurogné K, Kyndt F, Ali ME, Rogers TB, Lederer WJ, Escande D, Le Marec H, Bennett V (2003) Ankyrin-B mutation causes type 4 long-QT cardiac arrhythmia and sudden cardiac death. *Nature* 421:634–639.
- Moldoveanu T, Gehring K, Green DR (2008) Concerted multi-pronged attack by calpastatin to occlude the catalytic cleft of heterodimeric calpains. *Nature* 456:404–408.
- Müller AL, Freed D, Dhalla NS (2013a) Activation of proteases and changes in Na<sup>+</sup>-K<sup>+</sup>-ATPase subunits in hearts subjected to ischemia-reperfusion. *J Appl Physiol* 114:351–360.
- Müller AL, Hryshko L V., Dhalla NS (2013b) Extracellular and intracellular proteases in cardiac dysfunction due to ischemia-reperfusion injury. *Int J Cardiol* 164:39–47.
- Murphy RM, Dutka TL, Horvath D, Bell JR, Delbridge LM, Lamb GD (2012) Ca<sup>2+</sup>-dependent Proteolysis of Junctophilin 1 and Junctophilin 2.
- Nelson WJ, Traub P (1983) Proteolysis of vimentin and desmin by the Ca<sup>2+</sup>-activated proteinase specific for these intermediate filament proteins. *Mol Cell Biol* 3:1146–1156.
- Neuhof C (2014) Calpain system and its involvement in myocardial ischemia and reperfusion injury. *World J Cardiol* 6:638.
- Neuhof C, Fabiunke V, Deibele K, Speth M, Möller A, Lubisch W, Fritz H, Tillmanns H, Neuhof H (2004) Reduction of myocardial infarction by calpain inhibitors A-705239 and A-705253 in isolated perfused rabbit hearts. *Biol Chem* 385:1077–1082.
- Neuhof C, Fabiunke V, Speth M, Möller A, Fritz H, Tillmanns H, Neuhof H, Erdogan A (2008) Reduction of myocardial infarction by postischemic administration of the calpain inhibitor A-705253 in comparison to the Na<sup>+</sup>/H<sup>+</sup> exchange inhibitor Cariporide® in isolated perfused rabbit hearts. *Biol Chem* 389:1505–1512.
- Neuhof C, Götte O, Trumbeckaite S, Attenberger M, Kuzkaya N, Gellerich F, Möller A, Lubisch W, Speth M, Tillmanns H, Neuhof H (2003) A novel water-soluble and cell-permeable calpain inhibitor protects myocardial and mitochondrial function in postischemic reperfusion. *Biol Chem* 384:1597–1603.
- Ni R, Zheng D, Xiong S, Hill DJ, Sun T, Gardiner RB, Fan G-C, Lu Y, Abel ED, Greer PA, Peng T (2016) Mitochondrial calpain-1 disrupts ATP synthase and induces superoxide generation in type-1 diabetic hearts: a novel mechanism contributing to diabetic cardiomyopathy. *Diabetes*:1–34.
- Norberg E, Gogvadze V, Vakifahmetoglu H, Orrenius S, Zhivotovsky B (2010) Oxidative modification sensitizes mitochondrial apoptosis-inducing factor to calpain-mediated processing. *Free Radic Biol Med* 48:791–797.
- Ong SB, Subrayan S, Lim SY, Yellon DM, Davidson SM, Hausenloy DJ (2010) Inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury. *Circulation* 121:2012–2022.
- Ono Y, Saido TC, Sorimachi H (2016) Calpain research for drug discovery: Challenges and potential. *Nat Rev Drug Discov* 15:854–876 Available at: <http://dx.doi.org/10.1038/nrd.2016.212>.
- Ono Y, Sorimachi H, Suzuki K (1998) Structure and physiology of calpain, an enigmatic protease. *Biochem Biophys Res Commun* 245:289–294.
- Ozaki T, Nakazawa M, Yamashita T, Sorimachi H, Hata S, Tomita H, Isago H, Baba A, Ishiguro S ichi (2012) Intravitreal injection or topical eye-drop application of a  $\mu$ -calpain C2L domain peptide protects against photoreceptor cell death in Royal College of Surgeons' rats, a model of retinitis pigmentosa. *Biochim Biophys Acta - Mol Basis Dis* 1822:1783–1795.
- Ozaki T, Tomita H, Tamai M, Ishiguro SI (2007) Characteristics of mitochondrial calpains. *J Biochem* 142:365–376.
- Ozaki T, Yamashita T, Ishiguro S ichi (2008) ERp57-associated mitochondrial  $\mu$ -calpain truncates apoptosis-inducing factor. *Biochim Biophys Acta - Mol Cell Res* 1783:1955–1963.
- Ozaki T, Yamashita T, Ishiguro S ichi (2009) Mitochondrial m-calpain plays a role in the release of truncated apoptosis-inducing factor from the mitochondria. *Biochim Biophys Acta - Mol Cell Res* 1793:1848–1859.
- Ozaki T, Yamashita T, Ishiguro SI (2011) Ca<sup>2+</sup>-induced release of mitochondrial m-calpain from outer membrane with binding of calpain small subunit and Grp75. *Arch Biochem Biophys* 507:254–261.
- Pedrozo Z, Sánchez G, Torrealba N, Valenzuela R, Fernández C, Hidalgo C, Lavandero S, Donoso P (2010) Calpains and proteasomes mediate degradation of ryanodine receptors

- in a model of cardiac ischemic reperfusion. *Biochim Biophys Acta - Mol Basis Dis* 1802:356–362 Available at: <http://dx.doi.org/10.1016/j.bbadis.2009.12.005>.
- Penela P, Inverte J, Ramos P, Rodriguez-Sinovas A, Garcia-Dorado D, Mayor F (2019) Degradation of GRK2 and AKT is an early and detrimental event in myocardial ischemia/reperfusion. *EBioMedicine* 48:605–618 Available at: <https://doi.org/10.1016/j.ebiom.2019.09.019>.
- Penna C, Angotti C, Pagliaro P (2014) Protein S-nitrosylation in preconditioning and postconditioning. *Exp Biol Med* 239:647–662.
- Poncelas M, Inverte J, Aluja D, Hernando V, Vilarrosa U, Garcia-Dorado D (2017) Delayed, oral pharmacological inhibition of calpains attenuates adverse post-infarction remodelling. *Cardiovasc Res* 113:950–961.
- Potz BA, Sabe AA, Elmhadh NY, Clements RT, Abid MR, Sodha NR, Sellke FW (2017) Calpain inhibition modulates glycogen synthase kinase 3 $\beta$  pathways in ischemic myocardium: A proteomic and mechanistic analysis. *J Thorac Cardiovasc Surg* 153:342–357 Available at: <http://dx.doi.org/10.1016/j.jtcvs.2016.09.087>.
- Randriamboavonjy V, Kyselova A, Fleming I (2019) Redox Regulation of Calpains: Consequences on Vascular Function. *Antioxidants Redox Signal* 30:1011–1026.
- Sadayappan S, Finley N, Howarth JW, Osinska H, Klevitsky R, Lorenz JN, Rosevear PR, Robbing J (2008) Role of the acidic N' region of cardiac troponin I in regulating myocardial function. *FASEB J* 22:1246–1257.
- Sanchis D, Llovera M, Ballester M, Comella JX (2008) An alternative view of apoptosis in heart development and disease. *Cardiovasc Res* 77:448–451.
- Santella L (1998) The role of calcium in the cell cycle: Facts and hypotheses. *Biochem Biophys Res Commun* 244:317–324.
- Santos DM, Xavier JM, Morgado AL, Solá S, Rodrigues CMP (2012) Distinct regulatory functions of calpain 1 and 2 during neural stem cell self-renewal and differentiation. *PLoS One* 7.
- Shan L, Li J, Wei M, Ma J, Wan L, Zhu W, Li Y, Zhu H, Arnold JMO, Peng T (2010) Disruption of Rac1 signaling reduces ischemia-reperfusion injury in the diabetic heart by inhibiting calpain. *Free Radic Biol Med* 49:1804–1814.
- Shinkai-Ouchi F, Koyama S, Ono Y, Hata S, Ojima K, Shindo M, DuVerle D, Ueno M, Kitamura F, Doi N, Takigawa I, Mamitsuka H, Sorimachi H (2016) Predictions of Cleavability of Calpain Proteolysis by Quantitative Structure-Activity Relationship Analysis Using Newly Determined Cleavage Sites and Catalytic Efficiencies of an Oligopeptide Array. *Mol Cell proteomics*:1–40.
- Shintani-Ishida K, Yoshida KI (2015) Mitochondrial m-calpain opens the mitochondrial permeability transition pore in ischemia-reperfusion. *Int J Cardiol* 197:26–32.
- Shiraha H, Glading A, Chou J, Jia Z, Wells A (2002) Activation of m-Calpain (Calpain II) by Epidermal Growth Factor Is Limited by Protein Kinase A Phosphorylation of m-Calpain. *Mol Cell Biol* 22:2716–2727.
- Shirasaki Y, Yamaguchi M, Miyashita H (2006) Retinal penetration of calpain inhibitors in rats after oral administration. *J Ocul Pharmacol Ther* 22:417–424.
- Siklos M, BenAissa M, Thatcher GRJ (2015) Cysteine proteases as therapeutic targets: Does selectivity matter? A systematic review of calpain and cathepsin inhibitors. *Acta Pharm Sin B* 5:506–519.
- Singh R, Brewer MK, Mashburn CB, Lou D, Bondada V, Graham B, Geddes JW (2014) Calpain 5 Is Highly Expressed in the central nervous system (CNS), carries dual nuclear localization signals, and is associated with nuclear promyelocytic leukemia protein bodies. *J Biol Chem* 289:19383–19394.
- Singh RB, Chohan PK, Dhalla NS, Netticadan T (2004a) The sarcoplasmic reticulum proteins are targets for calpain action in the ischemic-reperfused heart. *J Mol Cell Cardiol* 37:101–110.
- Singh RB, Dandekar SP, Elimban V, Gupta SK, Dhalla NS (2004b) Role of proteases in the pathophysiology of cardiac disease. *Mol Cell Biochem* 263:241–256.
- Singh RB, Dhalla NS (2010) Ischemia-reperfusion-induced changes in sarcolemmal Na<sup>+</sup>/K<sup>+</sup> +-ATPase are due to the activation of calpain in the heart. *Can J Physiol Pharmacol* 88:388–397.
- Singh RB, Hryshko L, Freed D, Dhalla NS (2012) Activation of proteolytic enzymes and depression of the sarcolemmal Na<sup>+</sup> +K<sup>+</sup> +-ATPase in ischemia-reperfused heart may be mediated through oxidative stress. *Can J Physiol Pharmacol* 90:249–260.
- Sonobe T, Akiyama T, Du CK, Zhan DY, Shirai M (2014) Contribution of calpain to myoglobin efflux from cardiomyocytes during ischaemia and after reperfusion in anaesthetized rats. *Acta Physiol* 210:823–831.
- Sorimachi H, Ishiura S, Suzuki K (1997) Structure and physiological function of calpains. *Biochem J* 328:721–732.
- Takeshita D, Tanaka M, Mitsuyama S, Yoshikawa Y, Zhang GX, Obata K, Ito H, Taniguchi S, Takaki M (2013) A new calpain inhibitor protects left ventricular dysfunction induced by mild ischemia-reperfusion in in situ rat hearts. *J Physiol Sci* 63:113–123.
- Thompson J, Hu Y, Lesnefsky EJ, Chen Q (2016) Activation of mitochondrial calpain and increased cardiac injury: beyond AIF release. *Am J Physiol Heart Circ Physiol* 310:H376–H384.
- Totzeck M, Korste S, Miinalainen I, Hendgen-Cotta UB, Rassaf T (2017) S-nitrosation of calpains is associated with cardioprotection in myocardial I/R injury. *Nitric Oxide - Biol Chem* 67:68–74 Available at: <http://dx.doi.org/10.1016/j.niox.2017.04.003>.
- Trumbeckaite S, Neuhof C, Zierz S, Gellerich FN (2003) Calpain inhibitor (BSF 409425) diminishes ischemia/reperfusion-induced damage of rabbit heart mitochondria. *Biochem Pharmacol* 65:911–916.
- Wang Y, Chen B, Huang CK, Guo A, Wu J, Zhang X, Chen R, Chen C, Kutschke W, Weiss RM, Boudreau RL, Margulies KB, Hong J, Song LS (2018) Targeting Calpain for Heart Failure Therapy: Implications From Multiple Murine Models. *JACC Basic to Transl Sci* 3:503–517.
- Wang Y, Lopez D, Davey PG, Cameron DJ, Nguyen K, Tran J, Marquez E, Liu Y, Bi X, Baudry M (2016) Calpain-1 and calpain-2 play opposite roles in retinal ganglion cell degeneration induced by retinal ischemia/reperfusion injury. *Neurobiol Dis* 93:121–128.
- Wijnker PJM, Li Y, Zhang P, Foster DB, dos Remedios C, Van Eyk JE, Stienen GJM, Murphy AM, van der Velden J (2015) A novel phosphorylation site, Serine 199, in the C-terminus of cardiac troponin I regulates calcium sensitivity and susceptibility to calpain-induced proteolysis. *J Mol Cell Cardiol* 82:93–103.
- Ye T, Wang Q, Zhang Y, Song X, Yang D, Li D, Li D, Su L, Yang Y, Ma S (2015) Over-expression of calpastatin inhibits calpain activation and attenuates post-infarction myocardial remodeling. *PLoS One* 10:1–14.
- Yoshida K, Sorimachi Y, Fujiwara M, Hironaka K (1995) Calpain Is Implicated in Rat Myocardial Injury After Ischemia or Reperfusion. *Jpn Circ J* 59.
- Yoshida M, Suzuki A, Shimizu T, Ozawa E (1992) Proteinase-sensitive sites on isolated rabbit dystrophin. *J Biochem* 112:433–439.

- Yoshikawa Y, Hagihara H, Ohga Y, Nakajima-Takenaka C, Murata KY, Taniguchi S, Takaki M (2005) Calpain inhibitor-1 protects the rat heart from ischemia-reperfusion injury: Analysis by mechanical work and energetics. *Am J Physiol - Hear Circ Physiol* 288:1690–1699.
- Yoshikawa Y, Zhang GX, Obata K, Ohga Y, Matsuyoshi H, Taniguchi S, Takaki M (2010) Cardioprotective effects of a novel calpain inhibitor SNJ-1945 for reperfusion injury after cardioplegic cardiac arrest. *Am J Physiol - Hear Circ Physiol* 298.
- Zatz M, Starling A (2005) Calpains and Disease. *N Engl J Med* 352:2413–2423.
- Zhang CM, Gao L, Zheng YJ, Yang HT (2012) Berbamine protects the heart from ischemia/reperfusion injury by maintaining cytosolic Ca<sup>2+</sup> homeostasis and preventing calpain activation. *Circ J* 76:1993–2002.
- Zhang Y, Wang Y, Xu J, Tian F, Hu S, Chen Y, Fu Z (2019) Melatonin attenuates myocardial ischemia-reperfusion injury via improving mitochondrial fusion/mitophagy and activating the AMPK-OPA1 signaling pathways. *J Pineal Res* 66:0–3.
- Zhang Z, Biesiadecki BJ, Jin JP (2006) Selective deletion of the NH<sub>2</sub>-terminal variable region of cardiac troponin T in ischemia reperfusion by myofibril-associated  $\mu$ -calpain cleavage. *Biochemistry* 45:11681–11694.
- Zima A V., Blatter LA (2006) Redox regulation of cardiac calcium channels and transporters. *Cardiovasc Res* 71:310–321.