Regulatory role of calpain in neuronal death

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Abstract
Calpains are a group of calcium-dependent proteases that are over activated by increased intracellular calcium levels under pathological conditions. A wide range of substrates that regulate necrotic, apoptotic and autophagic pathways are affected by calpain. Calpain plays a very important role in neuronal death and various neurological disorders. This review introduces recent research progress related to the regulatory mechanisms of calpain in neuronal death. Various neuronal programmed death pathways including apoptosis, autophagy and regulated necrosis can be divided into receptor interacting protein-dependent necroptosis, mitochondrial permeability transition-dependent necrosis, pyroptosis and poly (ADP-ribose) polymerase 1-mediated parthanatos. Calpains cleave series of key substrates that may lead to cell death or participate in cell death. Regarding the investigation of calpain-mediated programmed cell death, it is necessary to identify specific inhibitors that inhibit calpain mediated neuronal death and nervous system diseases.

Key Words: nerve regeneration; calpain; calpastatin; central nervous system; apoptosis; autophagy; B-cell lymphoma; cyclin-dependent kinases; mitochondrial permeability transition; neural regeneration

Introduction
Three types of cell death have been identified (Green and Llambi, 2015; Chemaly et al., 2017). The first is apoptosis characterized by nuclear and cell shrinkage, chromatin condensation and fragmentation, and the formation of apoptotic bodies (Green and Llambi, 2015; Chemaly et al., 2017). Two apoptotic mechanisms have been reported: the intrinsic and extrinsic pathways (Chemaly et al., 2017). The extrinsic pathway is initiated by the binding of cell-surface death receptors to extracellular ligands, resulting in the formation of a death-inducing signaling complex. The intrinsic pathway is mediated by intracellular signals in the inner mitochondrial membrane (Chemaly et al., 2017). Following apoptotic signaling, many apoptotic proteins, such as caspases and B-cell lymphoma 2 (Bcl-2) family members, are activated or inhibited to participate in the regulation of apoptosis (Green and Llambi, 2015; Chemaly et al., 2017). The second type of cell death is autophagy, defined as the accumulation of two-membrane autophagic vacuoles in the cell plasma (Green and Llambi, 2015; Chemaly et al., 2017). The third type of cell death is necrosis, which features membrane rupture, release of cytoplasmic organelles, increased cytosolic calcium, and inflammation (Green and Llambi, 2015; Chemaly et al., 2017). In recent years, increasing evidence has indicated that necrosis can be molecularly controlled, and therefore it has been redefined as “regulated necrosis” (Galluzzi et al., 2014; Pasparakis and Vandenabeele, 2015). Regulated necrosis can be divided into cell-death modalities, such as receptor interacting protein (RIP)-dependent necroptosis, mitochondrial permeability transition (MPT)-dependent necrosis, and pyroptosis and poly (ADP-ribose) polymerase 1 (PARP1)-mediated parthanatos, which function in the pathogeneses of many nervous system diseases and during acute cellular damage (Pasparakis and Vandenabeele, 2015; Xiong et al., 2016; Che-
combined with calcium, is activated in the presence of phospholipids. Finally, the activated calpain degrades substrate proteins, such as spectrin, calcium-dependent transcription factor, caspase family members, Bcl-2 family members, RIP, and AIF, at membranes or in the cytosol after release from the membranes (Singh et al., 2014; Suzuki et al., 2014). Although calpain cleaves preferred sequences in association with preferred tertiary structures of substrates, the substrate specificity defies complete classification (Lynch and Gleichman, 2007). The complex involvement of calpains in vital cell functions suggests that the dysfunction of calpain may cause the excessive degradation or accumulation of cellular proteins, leading to various cellular damage and pathological conditions (Bartus, 1997, Suzuki et al., 2004). In this review, we summarize the current knowledge of the two main calpain isoforms, calpain-1 and calpain-2, in relation to different models of cell death and the roles of calpains with their specific substrates, which are important for the induction or repression of different models of cell death, such as apoptosis, autophagy and regulated necrosis.

**Calpain and Apoptosis**

**Calpain and caspase family members**

Caspase-dependent apoptosis is one of the main causes of neuronal death in neurodegeneration (Wang et al., 2016c; Ge et al., 2017). Calpains affect a number of proteins in the caspase family (Bakshi et al., 2005). For example, it has been widely verified that the activation of caspase-12 is mediated by calpains in the nervous system (Martinez et al., 2010; Imai et al., 2014). During neuronal death induced by salinomycin, activated calpain-1 and calpain-2 cleave and activate caspase-12, then activate caspase-9 and its effector protein caspase-3 (Gorman et al., 2000; Boehmerle and Endres, 2011). Calpains also mediate the activity of caspase-3 through other pathways. Yamada et al. (2012) reported that calpain-1 knockout neurons had less caspase-3 and apoptosis activity than heterozygous neurons, possibly because calpain-1 knockout increased the activity of X-linked inhibitor of apoptosis protein (XIAP), a physiological inhibitor of caspase-3. XIAP is degraded by calpain-1, and calpain-1 deficiency enhanced the inhibitory effect of XIAP on caspase-3 (Yamada et al., 2012). Although many researchers agree that calpains enhance caspase activity, other studies claim that calpain-2 can also cleave and block the activation of caspases in different cell types (such as MCF-7 cells and SH-SY5Y cells); furthermore, various effects have been reported in the same cell types under different apoptotic stimulations (such as staurosporine, hydrogen peroxide and serum starvation) (Chua et al., 2000; Tan et al., 2006). Therefore, calpain-2 may act as a negative regulator of caspase processing and apoptosis (Chua et al., 2000). Chua et al. (2000) reported that activated calpain-2 cleaved caspase-9, which is incapable of activating caspase-3, and prevented subsequent cytochrome c release in cells. In that study, the short pro-domain effectors, caspase-7/8/9, were calpain-specific cleavage sites (Chua et al., 2000). In summary, it is possible that calpains have opposite roles in apoptosis and their effects on caspases may be different (Wang et al., 2012).

**Effect of calpain on Bcl-2 family members**

Bcl-2 protein was first discovered by the analysis of chromosomal translocation in a B cell follicular lymphoma (Youle and Strasser, 2008; Kvensakul and Hinds, 2015; Wu et al., 2016). Subsequently, other Bcl-2 family proteins, such as Bak, Bid, and Bcl-xl, were identified (Kvensakul and Hinds, 2015). The Bcl-2 family proteins are essential in the mitochondrial apoptotic pathway, because they directly regulate the permeability of the outer mitochondrial membrane. A channel is formed on the outer mitochondrial membrane by Bax and Bad, through which cytochrome c, apoptosis protease activating factor 1, and the deoxyribonucleotide triphosphate complex, enter the cytoplasm, which activates caspase-9 and caspase-3 and induces apoptosis (Bleicken et al., 2013). Activated calpains cleave the N-terminal of Bax into a pro-apoptotic 18-kDa fragment, stimulating the release of cytochrome c and apoptosis (Gao and Dou, 2000). Similarly, under the stimulus of apoptotic signals such as Shigella infection, ischemia/reperfusion injury, and DNA-damaging agents, calpain-1 splices Bid into t-Bid, which has a better binding affinity for mitochondrial membranes, increases membrane permeability and produces oligomers that regulate apoptosis (Chen et al., 2001; Mandic et al., 2002; Andree et al., 2014).

**Effect of calpain on AIF**

AIF translocates from the mitochondria to the cytoplasm and into the nucleus when exposed to apoptotic signaling (Sevrioukova, 2011). In the inner membrane of mitochondria, AIF is truncated by calpains and this truncated AIF (tAIF) enters the cytoplasm through a permeability transition pore. It then activates caspase-9 and induces the endogenous apoptotic pathway by initiating chromatin condensation and DNA fragmentation (Ghavami et al., 2014). Yamada et al. (2012) reported that during neuronal apoptosis induced by ischemia, the translocation of AIF from the mitochondria to the cytosol was decreased in calpain-1 knockout neurons. As a result, apoptosis was inhibited. Similarly, in retinitis pigmentosa, the inhibition of calpain-1 inhibited AIF activation and decreased retinal degeneration and photoreceptor apoptosis (Ozaki et al., 2013). Heat shock protein 70 is associated with AIF and sustains the stability of AIF to prevent apoptosis; however, calpain-1 degrades heat shock protein 70 to maintain the transport of AIF from the mitochondria to the cytoplasm or nucleus (Matsumori et al., 2005).

**Effect of calpain on cyclin-dependent kinase 5 (CDK5)**

Cyclin-dependent kinases (CDKs) are a family of protein kinases first discovered for their roles in regulating the cell cycle (Bramanti et al., 2015). They are also involved in regulating transcription, apoptosis, and the differentiation of nerve cells (Arisan et al., 2014; Bramanti et al., 2015). They are present in all known eukaryotes, and their regulatory function in the cell cycle has been evolutionarily conserved (Bramanti et al., 2015). A CDK binds to cyclin, a regulatory protein. Without cyclin, CDK has little kinase activity; only the cyclin-CDK complex is an active kinase. Therefore, the activity of CDKs is regulated by phosphorylation and by binding inhibitory proteins termed cyclin-dependent kinase inhibitors (Bramanti et al., 2015). Inactive CDK5 monomer is only functional when attached to regulatory subunit P35 or P25. P35 is hydrolyzed by calpain into P25 and P10. P25 activates CDK5, forming a CDK5-P25 complex, which in-
activates myocyte enhancer factor, an important survival factor for dopamine neurons (Mount et al., 2013; Zhang et al., 2016). The CDK5-P25 complex upregulates P53 expression, which activates caspase-3 and induces apoptosis (Alvira et al., 2008). Furthermore, the phosphorylation of NR2A, a subunit of N-methyl-D-aspartate receptors (NMDAR), is increased by the calpain-P35/P25-CDK5 pathway, which leads to the increased expression of functional NRMR and calcium overload, resulting in glutamate-induced retinal neuronal apoptosis (Miao et al., 2012).

Calpain and Autophagy

Autophagy is a physiological process that digests extra substances in the cytoplasm by the autophagosome lysosomal pathway (Yang and Klionsky, 2010; Ohsumi, 2014). The critical role of autophagy is removing damaged intracellular organelles/misfolded proteins in neurons (Yang and Klionsky, 2010; Ohsumi, 2014). A number of studies have indicated that calpains interfere with autophagic pathways in the nervous system (Williams et al., 2008; Zhang et al., 2009; Menzies et al., 2015). Ataxin-3, the disease protein in Machado-Joseph disease, was predominantly hydrolyzed by calpain-1/2 (Weber et al., 2017). The disturbance of calpain-1/2 inhibition might promote the formation of ataxin-3 (Hubener et al., 2013), ultimately leading to the inhibition of autophagy (Watchon et al., 2017). In recent years, more than 36 subtypes of autophagy-related genes (Atg) involved in autophagy have been identified (Ohsumi, 2014). The early formation of autophagosomes requires multiple Atg complexes and Beclin-1 (Russo et al., 2011; Chiney et al., 2014). Based on a study by Chiney et al. (2014), after retinal injury, Atg-5 is inactivated by calpain-1, which attenuates autophagic activity in photoreceptor neurons (Chiney et al., 2014). Furthermore, calpains degrade beclin-1 and inhibit autophagy (Russo et al., 2011). Further studies indicated that under some autophagic conditions, reduced intracellular calcium levels might serve as a signal to inhibit calpain-1 activity, which in turn might activate autophagic activity by enhancing the levels of autophagic signaling molecules such as Atg-5, beclin-1 and the Atg-12-Atg-5 complex (Cecconi and Levine, 2008). Lysosome rupture is an essential element in cell death (Rodriguez-Muela et al., 2015). A previous study reported that permeability of the lysosome membrane is regulated by calpain by many mechanisms (Geronimo-Olvera et al., 2017). Calpain cleaves lysosome-associated membrane protein 2 at the lysosome membrane and mediates lysosomal membrane permeabilization, which may lead to lysosomal dysfunction and decreased autophagy (Villalpando Rodriguez and Torriglia, 2013; Rodriguez-Muela et al., 2015; Geronimo-Olvera et al., 2017).

Studies have also suggested that calpains are responsible for the conversion of the autophagic pathway to the apoptotic pathway (Yousefi et al., 2006; Chung et al., 2015; Song et al., 2017). During cell death in hippocampal neural stem cells induced by insulin withdrawal, the inhibition of calpain-2 leads to a preference for autophagic cell death, and an increase of calpain-2 expression converts the autophagic pathway to the apoptotic pathway (Chung et al., 2015). This finding indicates that autophagy might have a close connection with apoptosis, as autophagy influences apoptosis through the degradation of caspase-8 or -9. Furthermore, apoptosis affects the autophagic flux by cleaving autophagy molecules, such as Beclin 1 or Atg-5 (Chung et al., 2015). Other studies reported that when Atg-5 is cleaved by calpain its autophagy activity is inhibited (Yousefi et al., 2006; Del Bello et al., 2013). In cells exposed to apoptotic stimuli, cleaved Atg5 translocates into the mitochondria and combines with Bcl-xL, thereby inducing apoptosis (Zhou et al., 2011; Del Bello et al., 2013).

Calpain and Regulated Necrosis

Calpain and parthanatos

A series of genotoxic stresses, such as alklyating agents and N-methyl-N-nitro-N-nitrosoguanidine, have been verified to result in cell necrosis associated with the activation of PARP-1, termed parthanatos (Wang et al., 2009; Harbison et al., 2011; Muller et al., 2014). Parthanatos characterized by overactive PARP-1 is caused by metabolic disturbance, such as the excessive consumption of nicotinamide adenine dinucleotide and adenosine triphosphate (van Wijk and Hageman, 2005). Baritaud et al. (2010) further uncovered the specific molecular mechanism by which calpains regulate necrosis. Acute DNA damage activates PARP-1 in the nucleus. When activated, PARP-1 is transferred to the mitochondrial membrane to activate calpain-1, which is truncated and activates AIF. In the nucleus, AIF, Histone H2AX and cyclophilin break down the DNA into large fragments (Baritaud et al., 2010). Calpain-1 mediates AIF release from mitochondria and necrosis through a mechanism that is distinct from apoptosis but which is caspase-independent. The reason might be explained, because apoptosis is abrogated by the cleavage of apoptotic effectors, such as caspases, by calpain (Moubarak et al., 2007). Furthermore, because calpain-1 directly cleaves AIF to tAIF, calpains might also cleave BID to t-BID, which facilitates BAX activation and subsequent AIF mediated parthanatos (Cabon et al., 2012). In addition, PARP-1 is the substrate of calpains and the activation of PARP-1 requires the activation of calpains (Sacca et al., 2016). Therefore, PARP-1 and calpain-1 might act in concert following injury to induce AIF-mediated necrosis (Chiu et al., 2012).

Calpain and necroptosis

Necroptosis, a RIP-mediated programmed form of necrosis induced by tumor necrosis factor α, is regulated by calpain (Bollino et al., 2015; Pasparakis and Vandenabeele, 2015) (Figure 1). Calpains degrade c-Jun N-terminal kinase (JNK) inhibitor JNK-interacting protein-1, and then activate JNK-1, which increases the expression of RIP-1. RIP-1 then binds to RIP-3 and initiates necroptosis mechanisms, including second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI and AIF release from mitochondria (Bollino et al., 2015). Our previous studies reported that in vitro elevated hydrostatic pressure or oxygen-glucose deprivation induced the necroptosis of retinal ganglion cells (RGC-5 cell line); thus, calpains play an important role in mediating necroptosis via tAIF (Shang et al., 2014; Chen et al., 2016). Our recent research also found that Pin1 interacts with calpastatin, an endogenous calpain inhibitor, to modulate the activity of calpain 2 in the presence of excessive glutamate, thereby causing tAIF mediated necroptosis in primary cells.
rat retinal neurons, the RGC-5 cell line, and the ganglion cell layer and inner nuclear layer of the rat retina (our unpublished data). Although necroptosis and parthanatos share a common necrotic effector, AIF, they represent two independent and distinct pathways that regulate necrosis (Sosna et al., 2014). Necroptosis and parthanatos are induced by tumor necrosis factor α and N-methyl-N-nitro-N-nitrosoguanidine, respectively. In contrast to parthanatos that does not depend on caspase activity, the regulation of necroptosis depends on caspase-8 activity (Sosna et al., 2014).

**Calpain and other regulated necroses, including pyroptosis and MPT mediated regulated necrosis**

Pyroptosis is a form of inflammatory cell necrosis that requires the activation of caspase-1 (Fink and Cookson, 2005; He et al., 2015). During pyroptosis, caspase-1 is activated by the pyroptosome, which is composed of dimers of the adapter protein apoptosis-associated speck protein containing a CARD or caspase activation and recruitment domain (Soong et al., 2012; He et al., 2015). Although there are numerous caspase-1 activation pathways, the downstream pathway results in pyroptosis; the apoptosis pathway is associated with caspases-3 and -7, but not caspase-1 (Soong et al., 2012; Sun et al., 2016). There are two types of sensory receptors involved in pyroptosis, Toll-like receptors and Nod-like receptors, which sense danger signals (Soong et al., 2012). Chun et al. (2009) reported that Toll-like receptor 2 stimulation results in increased calcium flux. Subsequently, the activation of calcium dependent calpains is targeted by caspase-1, which cleaves the transmembrane proteins occludin and E-cadherin (Chun and Prince, 2009; Soong et al., 2012), resulting in pyroptosis.

The MPT pore is an inducible inner mitochondrial membrane pore involved in apoptotic and necrotic death (Douglas and Baines, 2014; Lu et al., 2014). The formation of MPT is regulated by calpain-mediated proteolytic events (Arrington et al., 2006). Under MPT conditions, osmosis forces a large volume of water into the mitochondrial matrix, resulting in the release of various apoptotic activators such as Bcl-2 family members, into the cytoplasm (Oh and Lim, 2006). MPT also triggers a pathway that regulates necrosis and which is regulated by a key regulatory molecule, cyclophilin D (Lu et al., 2014). Subsequent studies found that MPT formation is an important upstream mediator of integrin α<sub>6</sub>β<sub>1</sub> inactivation and that calpain activation might activate integrin through talin cleavage (Liu et al., 2013).

However, although pyroptosis and MPT regulated necrosis were confirmed in neurons, correlations between calpain and the two types of necrosis are lacking. Therefore, studies are needed to determine whether calpain has a relationship with cell necrosis in nervous system diseases.

**Perspective**

In addition to the above mentioned substrates, other studies reported that the direct targets of calpains include most major glutamate receptors, such as α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors, NMDA receptors, and metabotropic glutamate receptors (Dong et al., 2004; Wu et al., 2007; Curcio et al., 2016; Wang et al., 2018). By the proteolysis of these receptors and associated proteins, calpains may modulate the activity of glutamate synapses (Curcio et al., 2016). As a result, calpain proteolysis in neurons might result in pathological events such as excitotoxicity, but also neuroprotective roles in cell survival and synaptic transmission (Wu et al., 2007; Doshi and Lynch, 2009). Calpain-1 and calpain-2 play opposite roles in cell survival and death (Wang et al., 2016b). Activation of synaptic NMDAR-coupled calpain-1 is neuroprotective, while activation of extrasynaptic NMDAR-coupled calpain-2 is neurodegenerative (Wang et al., 2016b). Calpain-1 is involved in Akt and extracellular signal-regulated kinase mediated cell survival. Activated calpain-2 cleaves and inactivates STEP, resulting in p38-induced cell death (Wang et al., 2016b). This provides new insights into the mechanism of calpain mediated cell death. Based on these previous findings, further studies are needed to explore the detailed mechanisms of calpain mediated cell survival or death.

For most calpain mediated disorders, inhibitors are the first and logical therapeutic choice (Ono et al., 2016). To achieve a potential therapy, it is critical to produce specific molecules that have the correct physical chemistry to function as drugs for the treatment of neural diseases (Bartus et al., 1999; Lauer and McIntosh, 2001; Wang et al., 2016a). Many previous studies have suggested that calpain inhibitors are useful for treating brain injuries by preventing neuronal loss and improving behavior (Saatman et al., 1996b; Bartus, 1997; James et al., 1998; Marklund et al., 2006; Pandey et al., 2016). Furthermore, some calpain inhibitors are being tested in clinical trials; AbbVie has initiated a Phase I clinical trial with an orally active non-selective calpain inhibitor for the treatment of Alzheimer’s disease (Wang et al., 2016b). However, to our knowledge, many of the calpain inhibitors are nonspecific and target other proteases. Therefore, understanding the calpain substrates and the specific pathways of calpain mediated cell death are key concerns, especially when considering potential off-target effects (Ono et al., 2016). There is also a need to develop specific and beneficial therapeutic calpain inhibitors.

**Conclusions**

A preliminary understanding of the regulatory role of calpains in programmed neuronal death has been reached. Calpains play an important role in apoptosis, autophagy, and regulate necrosis (Table 1). Therefore, calpain is a promising therapeutic target for neurological diseases. Specific inhibitors of calpains may bring new insights for the treatment of related diseases. However, because of potential species-specific differences, there have been few investigations regarding the mechanisms involved in human nervous system diseases, which are different from cells or animal models of disease. Therefore, different potential risks, including calpain dysfunction, might lead to the progression of one type of nervous disease. Thus, future pathogenic mechanisms, not only the deregulated calpain mediated central nervous system dysfunction, should be investigated further to understand the progression of nervous diseases.

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Figure 1 Hypothetical mechanisms of calpain in neuronal death.

LAMP, Ataxin, and the complex of Beclin and Atg are activated by inactivated calpain, which inhibits autophagy. The inactivated complex of Beclin and Atg, induced by activated calpain, converts autophagy to apoptosis. Calpain is implicated in numerous steps during apoptosis, including the cleavage and activation of caspases, which lead to the release of cytochrome c and AIF. Hsp70 physiologically stabilizes AIF. Calpain also cleaves Bcl-2 family members, including Bax and Bid, to promote apoptosis and promotes the formation of CDK5-P25 complex, which also promotes apoptosis. Except for AIF mediated apoptosis, AIF combined with H2AX, mediated by PARP-1, might lead to regulated necrosis. In addition, calpain cleaves a series of substrates, such as JNK-interacting protein-1, and activates proteins, including integrin and RIP-1, to promote necrosis.

Table 1 Specific substrates of calpains that induce or repress cell death

| Cell-death types | Key death pathway | Death regulatory factors |
|------------------|-------------------|-------------------------|
| Apoptosis        | Caspase family    | Caspase-3/7/8/9/12, XIAP|
|                  | Bcl-2 family      | Bax, Bid                |
|                  | Apoptosis induced | AIF, HSP70              |
|                  | factor             |                         |
|                  | Cyclin-dependent  | CDK5, P35, NR2A          |
|                  | kinase             |                         |
| Autophagy        | Autophagy-related | Ataxin-3, Atg-5/12,     |
|                  | genes              | beclin-1, LAMP2          |
| Regulated        | Parthanatos        | PARP-1, AIF, BID         |
| necrosis         | Necroptosis        | JNK-interacting protein-1, AIF |
|                  | Pyroptosis         | Caspase-1, occluding, E-cadherin |
|                  | MPT-mediated necrosis | MPT, cyclophilin D, αmβ3 |

Bcl-2: B-cell lymphoma 2; MPT: mitochondrial permeability transition; XIAP: X-linked inhibitor of apoptosis protein; AIF: apoptosis induced factor; HSP70: heat shock protein 70; CDK5: cyclin-dependent kinase 5; Atg: autophagy-related; LAMP2: lysosome associated membrane permeabilization; PARP1: pyroptosis and poly (ADP-ribose) polymerase.

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