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Review

Conventional calpains and programmed cell death

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The evidence on the crucial role of a family of calcium-dependent cysteine proteases called calpains in programmed cell death is rich and still growing. However, understanding of the mechanisms of their functions in apoptosis is not full yet. Calpains have been implicated in both physiological and pathological cell death control, especially in various malignancies, but also in the immune system development and function. There is also growing evidence on calpain involvement in apoptosis execution in certain pathological conditions of the central nervous system, in cardiovascular diseases, etc. Understanding of the clinical significance of calpain activation pathways, after intense studies of the influence of calpain activity on drug-induced apoptosis, seems especially important lately, as calpains have become noticed as potential therapeutic targets. To allow pharmacological targeting of these enzymes, thorough knowledge of their patterns of activation and further interactions with already known apoptotic pathways is necessary. A comprehensive summary of both well established and recently obtained information in the field is an important step that may lead to future advances in the use of calpain-targeted agents in the clinic.

Keywords: calpain, apoptosis, cancer, neurodegeneration

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INTRODUCTION

The calpain family comprises calcium-activated, neutral, mainly cytosolic cysteine proteases. These enzymes are either expressed ubiquitously in all mammalian tissues, or tissue-specific, with especially well known skeletal muscle-specific calpain homologue, p94 (also called calpain 3) or nCL-2/calpain-8a identified as stomach-specific. The best characterized representatives of ubiquitously present calpains are two distinct, heterodimeric isoforms: μ- and m-calpains (also known as calpains I(1) and II(2)), called classical or conventional calpains. The μ- and m- prefixes in the common names of these enzymes directly correspond to their calcium (Ca^{2+}) requirements for activation *in vitro* — respectively micro- and millimolar. The conventional calpains, being heterodimers, consist of two distinct subunits: a 28-kDa regulatory subunit (identical for both enzymes) and an 80-kDa catalytic subunit (sharing 55–65% sequence homology between them). Both enzymes cleave numerous cellular proteins, the current number of those reaching over a hundred, and thus play crucial roles at probably all stages of cellular existence — from proliferation and differentiation, to aging and death. Calpain substrates comprise several enzymes, such as protein kinase C (PKC) and poly(ADP-ribose) polymerase (PARP), various signal transducers and transcription factors (e.g. c-fos, c-jun, nuclear factor-κB (NFκB) together with its inhibitor IκB, STAT5, STAT3), cytoskeletal proteins (talin, fodrin), numerous adhesion molecules, and multiple others (Hirai *et al.*, 1991; Saido *et al.*, 1994; Sorimachi *et al.*, 1997; Suzantova *et al.*, 1999; Oda *et al.*, 2002; Perrin *et al.*, 2002; Goll *et al.*, 2003). Moreover, calpains regulate their own function by limited autoproteolytic cleavage, thus reducing their Ca^{2+} requirements for activity (Saido *et al.*, 1994; Sorimachi *et al.*, 1997; Suzantova *et al.*, 1999; Perrin *et al.*, 2002; Goll *et al.*, 2003). The structural basis of substrate recognition by calpains is still not fully understood; however, strong preferences are observed, e.g. for so called PEST sequences — residues rich in proline (P), glutamic/aspartic acid (E), serine (S) and threonine (T) — or for some general patterns of both primary and secondary protein structure (Tompa *et al.*, 2004).

Probably the key to understanding the nature of calpains is that they do not exert the "digestive" type of proteolysis. It has to be strongly stressed here that the calpain-mediated protein cleavage is a limited process that rather modifies the target proteins (usually removing a peptide that is either blocking the substrate activity or is necessary for it) than destroys them, and that these modifications may have an overwhelming impact on the basic functions of targeted compounds (Saido *et al.*, 1994; Sorimachi *et al.*, 1997; Suzantova *et al.*, 1999; Perrin *et al.*, 2002; Goll *et al.*, 2003). For instance the calpain-mediated modification is supposed to be an important element of calcineurin autoinhibitory domain proteolytic cleavage resulting in triggering its phosphatase activity. Other well documented examples are regulation of adhesion complexes by calpain, that is required for cell migration and motility, and calpain-mediated NMDA receptor truncation believed to be a key factor for feed-back regulation of the receptor activity (Bi *et al.*, 1998; Franco *et al.*, 2004; Liu *et al.*, 2005). There is also growing evidence for an important role of calpain in signal transduction *via* the generation of cAMP through G-protein-coupled receptors by proteolytic activation of Gs_{α} (Sato-Kusubata *et al.*, 2000). Removal of the N-terminal region from tyrosine hydroxylase by calpain, resulting in disruption of the quaternary structure, changes its susceptibility to physiological regulatory mechanisms (Kiuchi *et al.*, 1991). Such cases are numerous.

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Abbreviations: AIF, apoptosis inducing factor; B-CLL, B-cell chronic lymphocytic leukemia; cdk, cyclin-dependent kinases; CNS, central nervous system; DXR, doxorubicin; PARP, poly(ADP-ribose) polymerase; PS, phosphatidylserine; STS, staurosporine; XIAP, X-linked inhibitor of apoptosis protein

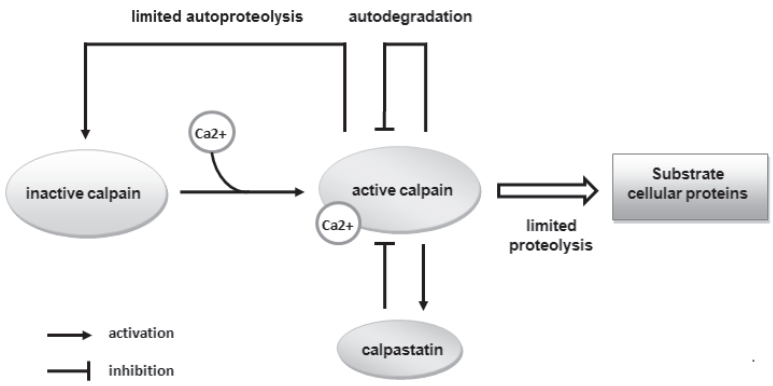

Figure 1. Calpains are activated by elevated Ca^{2+} and enhance their own activity through limited autoproteolytic cleavage
This activity may be then restricted by both autodegradation (limited autoproteolysis) and inhibitory effect of calpastatins. The latter have to be first activated by calpain-mediated limited proteolysis. All those processes are strictly calcium-dependent.

The complex involvement of calpains in vital cell functions determines the need of tight control over the system. Uncontrolled calpain-mediated proteolysis of cellular proteins is prevented by calpastatins, the only endogenous protein inhibitors that are exclusively specific for ubiquitous calpains, whereas other cellular proteins capable of blocking calpain activity — e.g. L- and H-kininogens — seem to exhibit little or no calpain-directed specificity. At the same time calpastatins are probably one of the most crucial calpain substrates, as they require proteolytic modification by an active calpain to exert their inhibitory activity. This fact, together with the already mentioned autoproteolytic, self-regulatory function of calpains, suggests a highly sophisticated network of interdependencies, protecting the cell from uncontrolled calpain activation (Fig. 1) (Saido *et al.*, 1994; Sorimachi *et al.*, 1997; Suzantova *et al.*, 1999; Perrin *et al.*, 2002; Goll *et al.*, 2003).

Recently, conventional calpains have drawn growing attention especially in oncology-related research areas. As programmed cell death seems to be one of the most promising novel targets in drug research, any possible new factors influencing it seem to be very appealing for study.

Calpains, observed first in rat brain (Guroff, 1964) and then in skeletal muscle (Huston & Krebs, 1968), were first mentioned to participate in cell death only in 1993, supposedly being involved in *N*-methyl-1-D-aspartate (NMDA)- and ischemia-induced apoptosis (Roberts-Lewis *et al.*, 1993). Next, limited calpain self-cleavage to the active form was noted in thymocyte cell death after treatment with dexamethasone, and in metamyelocyte death, following incubation with cycloheximide; moreover, these apoptotic processes were noticed to diminish greatly after incubation with an exogenous calpain inhibitor (calpeptin) (Squier *et al.*, 1994). These observations opened a new and promising field of research in apoptosis, especially in the clinical context of experimental oncology. The role of calpains in regulation of tumor development and apoptosis in certain common human neoplasms — such as lung, breast, prostate and several other cancers — is now generally appreciated; however, it is still not always precisely understood (Braun *et al.*, 1999; Mamoune *et al.*, 2003). It seems indispensable to gain this comprehension and the only method is to track down the distinct moments in cellular control mechanisms that may be affected by those enzymes.

CALPAINS AND CASPASES

Caspases are nowadays universally recognized as the main players in the apoptosis scenario (Green, 2000; Danial & Korsmeyer, 2004), not surprisingly then a possible relationship of calpains to the observed increased activity of caspases in dying cells was searched for. In fact some striking similarities between those proteases could not have gone unnoticed. They share numerous substrates such as α-fodrin (non-erythroid α-spectrin), PARP, calmodulin-dependent protein kinase IV (CaMKIV) etc. The number of cellular proteins being dually susceptible to their proteolytic activity, sometimes, however, differing in cleavage specificity or site, is still growing. What is even more important, both types of enzymes are each other's substrates, which adds to the complexity of their possible interactions, especially if we note additionally that caspases are also able to cleave calpastatin, which in turn inhibits calpain activity (Wang, 2000). The data accumulated over years of intense research indicate with little doubt that caspases and calpains may work either in concert or independently and sometimes in opposition during cell death, even if the relation might at times appear to be rather subtle.

Probably the leading concept in the subject of their interactions is that calpains and caspases may sequentially influence cell death. In such configuration calpains may act as either pro- or anti-apoptotic (depending on the tissue specificity or external environment) upstream regulators of caspase processing (Khanna *et al.*, 1998; Ruiz-Vela *et al.*, 1999; Chua *et al.*, 2000; McCollum *et al.*, 2002). The other aspect of the phenomenon is the caspase-3-mediated degradation of endogenous calpain inhibitors, facilitating calpain activation and further proteolysis — sometimes, in the form of a positive feedback — of both caspases and calpains (Pörn-Ares *et al.*, 1998; Wang *et al.*, 1998; Kato *et al.*, 2000; Mikosik *et al.*, 2007); it is not impossible, however, for a caspase also to directly activate calpains during apoptosis (Wood & Newcomb, 1999).

First studies of the influence of calpains on caspases in apoptosis left an impression of the former being rather proapoptotic factors and exerting that effect through caspase processing. In 1998 the Waterhouse's group demonstrated primary activation of calpains during radiation-induced cell death resulting in caspase-3 activation and subsequent apoptosis (Khanna *et al.*, 1998). Only one year later Ruiz-Vela noted that calpain specifically triggered activation and cleavage of caspase-7 both *in vitro* and *in vivo*, being therefore responsible for B cell clonal deletion during their differentiation and specificity development (Ruiz-Vela *et al.*, 1999). Similar conclusions were brought about by research on endoplasmic reticulum stress- and ischemic injury-induced apoptosis, as calpain-dependent caspase-12 activation was then observed (Nakagawa & Yuan, 2000; Tan *et al.*, 2006).

During studies on drug-induced apoptosis in human neuroblastoma cell lines procaspase-3 was, indirectly

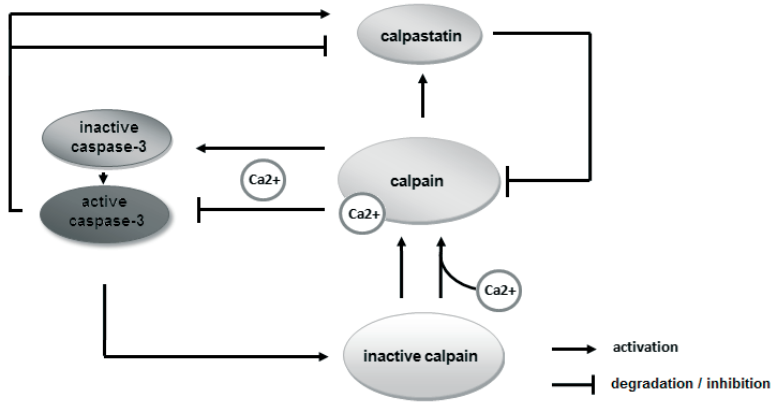

Figure 2. Calpains may exert both inhibitory and activating effects on caspase-3
Caspase-3 influences calpains both directly (*via* limited proteolysis of the enzyme) and indirectly (through calpastatin) leading to their activation or inhibition, depending on the tissue type as well as on the level and sequence of activation of both proteases.

though, shown to be a natural endogenous calpain substrate. In that case, contrastingly to much more popular observations mentioned above, cleavage of the procaspase lead to permanently inactivated, truncated form of the enzyme (McGinnis *et al.*, 1999), suggesting that caspase-3/calpain interactions' pattern may be highly tissue-dependent. Complex relations between caspase-3 and ubiquitous calpains are shown in Fig. 2.

Also, direct proteolysis of caspase-7, -8 and -9 by calpain II generated inactive fragments (Chua *et al.*, 2000); this observation lead to one of the very first ideas as to how calpain activity might directly hinder apoptotic processes in addition to intensifying them, as had been supposed and observed earlier. After years of indirect observations, what was especially important, the gathered data gave the first evidence of caspases being direct substrates of calpains. Chua and coworkers identified a set of calpain-specific cleavage sites in caspases, focusing first on the short prodomain effector caspase-7, and, after surprisingly promising preliminary results, examining also the long prodomain initiator caspases-8 and -9. Eventually, they proved a direct inhibitory function of calpain on caspases-9 (well known for its key role in mitochondrial apoptotic pathway), -7 and -8.

Ample evidence of calpain influence on caspases and *vice versa* has been accumulated since. Results of many studies gave support to the thesis that calpains participate in apoptosis in a highly diverse way. Summarizing, we know now that conventional calpains may both promote and inhibit apoptosis and their influence on caspases may also be different in various cell types, sometimes differing even in the same cell types under varying death stimuli, the last concept being presented and efficiently defended on calpain overexpressing or calpain gene-deficient cell lines (Lu *et al.*, 2002; Tan *et al.*, 2006).

Calpains and Bcl-2 protein family

Caspases are not the only group of cellular proteins involved in regulation and execution of programmed cell death, which is influenced by calpains. Another target of this activity of calpains consists of the Bcl-2 protein and its homologs. They exert control over apoptosis at its mitochondrial stage, playing an activating or inhibitory role, depending on the protein nature itself or the homo/heterodimeric form they can create with other members of the same family. The result of cleavage of their inactive precursors and their following dimerization is outer mitochondrial membrane permeabilization with consequent release of certain compounds sequestered within the organelle or between the inner and outer mitochondrial membranes. These compounds, e.g. cytochrome *c*, are indispensable in some pathways of apoptosis. Healthy cells express the whole spectrum of Bcl-2 proteins and the ratio of pro- and anti-apoptotic ones, the degree of their cleavage and temporary or constant dimerization can determine the cell death rate under different conditions (Reed, 1998; Burlacu, 2003; Chipuk & Green, 2008).

Calpains have often been implicated in the cleavage of a number of Bcl-2 family members. Direct interactions between calpains and Bcl-2 homologs were noticed quite early. In 1998, Wood and coworkers reported that purified calpain cleaved Bax in a calcium-dependent manner. Bax is probably the best-studied protein among the proapoptotic Bcl-2 family members. During apoptosis, the death signal causes Bax translocation into the mitochondria together with its dimerization and conformational change. Removal of the N-terminal 20 amino acids of Bax occurring prior to those is a factor enabling it to target mitochondria (Goping *et al.*, 1998). Active calpains have been shown to cleave Bax at its N-terminus (at Asp33), generating a potent proapoptotic 18-kDa fragment (Bax/p18). Moreover, unlike full-length Bax, Bax/p18 loses its ability to interact with the antiapoptotic Bcl-2 or Bcl-xL, which is supposedly a potent cellular pathway of avoiding apoptosis (Fig. 3).

The mechanism of calpain-dependent cleavage of Bax proves to be especially efficient in drug-induced apoptosis, as calpain inhibitors blocked or noticeably attenuated Bax-mediated apoptotic processes in cells treated with etoposide or other chemotherapeutics (Gao & Dou, 2000; Cao *et al.*, 2003). Similar observations have been made in neuron cell lines, where cotreatment of staurosporine (STS)-treated cells with a calpain inhibitor blocked STS-induced Bax cleavage, significantly retarding apoptosis execution (Choi *et al.*, 2001).

Calpain I involvement was also investigated in neutrophil apoptosis (both spontaneous and Fas-induced) demonstrating that pharmacological and physiological (calpastatin-dependent) inhibition of its enzymatic function may prevent cleavage of Bax into an 18-kDa fragment unable to interact with Bcl-xL, the result being disabled cytochrome *c* release from the mitochondria leading to subsequent blockade of caspase-3 activation and apoptosis execution. Furthermore, a clinical manifestation of the observed situation appears clearly recognizable in several neutrophilia-associated inflammatory diseases. Cystic fibrosis patients' neutrophils, for example, were demonstrated to contain markedly increased levels of calpastatin and significantly decreased amounts of the 80-kDa proform of calpain-1 compared with normal neutrophils, the observation corresponding strongly with earlier findings of a decreased cell death rate and functional Bax deficiency (Dibbert *et al.*, 1999; Altznau-

er *et al.*, 2004). What is even more clinically important, pretreatment with calpeptin, a calpain inhibitor, blocked chemotherapy (etoposide)-induced calpain activation, Bax cleavage, cytochrome *c* release, caspase-3 activation and subsequent apoptosis in human leukemic cell lines (Gao & Dou, 2000).

Bax, although probably the most deeply studied, is by no means the only Bcl-2 family member cleaved by calpain. There are numerous reports about calpains being able to also cleave Bid, yielding an active proapoptotic product similar to the one obtained through typical, caspase-mediated activation. The experimental, indirect results have been fully supported by direct observations of recombinant Bid cleavage *in vitro*. Bid was demonstrated to undergo calpain-mediated processing leading to mitochondrial permeabilization and apoptosis following myocardial ischaemia/reperfusion (Mandic *et al.*, 2002). It was also suggested to be cleaved by calpain during chemotherapy-induced cell death (Chen *et al.*, 2001), similarly to Bak, another proapoptotic Bcl-2 relative, activation of which by calpain is supposed to be an important step in doxorubicin-mediated cell death (Panaretakis *et al.*, 2002). It is quite likely that calpains may in future fill in numerous gaps observed in the mechanisms of caspase-independent regulation of Bcl-2 family members' activation in certain modes of treatment-related apoptosis of human neoplasms.

Other potential apoptosis-related calpain targets

Of course, caspases and Bcl-2 family proteins, though the most thoroughly studied, do not complete the list of points where calpains may be involved in cell death control. p53 protein is a well established cell death or survival decisions regulator, reacting especially intensely to DNA damage. Its functions may be seriously altered in cancer cells thus leading to survival of potentially harmful cells instead of triggering their removal (Benard *et al.*, 2003). Multiple studies report calpains as able to cleave and modify functions of p53, thus modulating the cellular apoptotic potential. Both calpain I and II can cleave p53, the degree of susceptibility to cleavage being different in various p53 mutants (Kubbutat & Vousden, 1997; Gonen *et al.*, 1997; Sedarous *et al.*, 2003; Del Bello *et al.*, 2007). Kubbutat and coworkers identified the cleavage site preferential for calpain within the N-terminus of p53; in most studies the cleavage result was p53 activation and stabilization, with variations dependent on the stimulus and conditions used (Kubbutat & Vousden, 1997; Del Bello *et al.*, 2007).

Calpains cleave also other p53 family transcription factors. p73 is especially important here, being another element of DNA damage response and thought to be crucial in tumorigenesis and some forms of apoptosis (Melino *et al.*, 2002; 2003). Calpain-mediated modifications of p73 can lead to stabilization of this protein and supposedly ensure proper concentration ratio of its different isoforms (Munarriz *et al.*, 2005). As p73 function is deregulated in numerous human malignancies, its assessment being utilized as both prognostic and predictive tool (Tannapfel *et al.*, 1999; Takahashi *et al.*, 2000; Pfeifer *et al.*, 2006), calpain-related modification of the protein is worthy to keep it in mind as potentially important for tumorigenesis. This relation requires attention, especially when cosidered that some of the neoplasms associated with p73 aberrations (such as gastrointestinal and prostatic malignancies) were noted also to display certain abnormalities of calpain expression or function; eventually, it might potentially enable easier diagnosis and more efficient treatment of such cancers.

Calpains are likewise involved in modifying other potent cell cycle controllers, thereby influencing directly the cell cycle dynamics and indirectly potential switch towards the cell death pathway. Among the affected proteins, cyclins and cyclin-dependent-kinases (cdk) are probably most crucial. An indirect influence of calpains on cdk-5 is suggested to affect neuronal cell death. Cdk-5 in the CNS is generally associated with a 35-kDa brain-specific activator p35, that after truncation to p25 activates the kinase. Conversion of p35 to p25 may be driven by calpain-dependent proteolysis and under certain conditions contributes to neuronal apoptosis (Kusakawa *et al.*, 2000; Lee *et al.*, 2000).

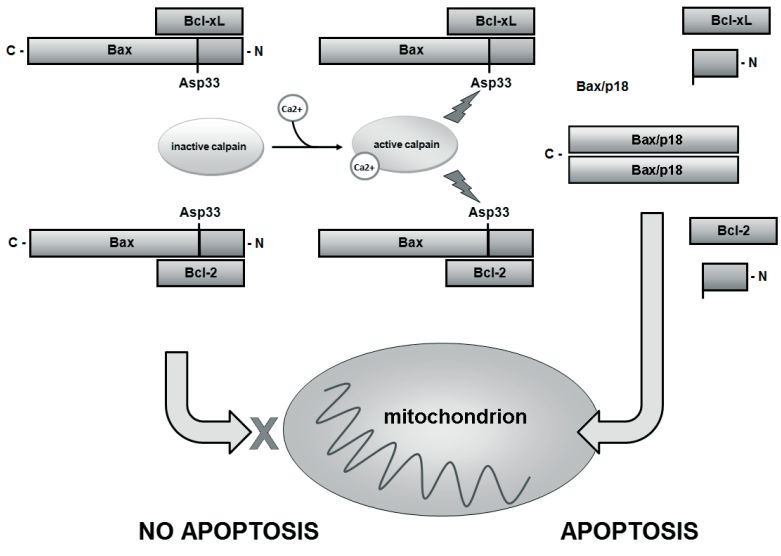

Figure 3. Calpain regulates Bax function
Active calpain cleaves proapoptotic Bax protein at Asp33, generating a truncated Bax/p18, and thus enabling it to homodimerize and enter mitochondria. Truncated form loses its ability to form heterodimers with antiapoptotic Bcl-2 family members.

Calpains cleave powerful intracellular apoptosis inhibitors, e.g. XIAP (X-linked inhibitor of apoptosis protein), thus constituting a formidable proapoptotic factor documented already in cardiovascular and nervous systems as well as in haematological disorders. XIAP has an inhibitory potential towards both initiator and effector caspases, especially a key apoptotic executioner — caspase-3. Reports on calpain-dependent, limited proteolysis and subsequent strong up-regulation of the latter enzyme in various tissues provide the mechanism for previously unexplained activating effect of calpain on caspases. The reported role of calpain in XIAP cleavage leads to further questions on higher levels of cellular death control in human organism in which calpains appear to play a vital role. The pathway: injury → calpain activation → XIAP degradation → caspase activity upregulation, seems to be an universal one, even if factors triggering it are somewhat varied, from degraded

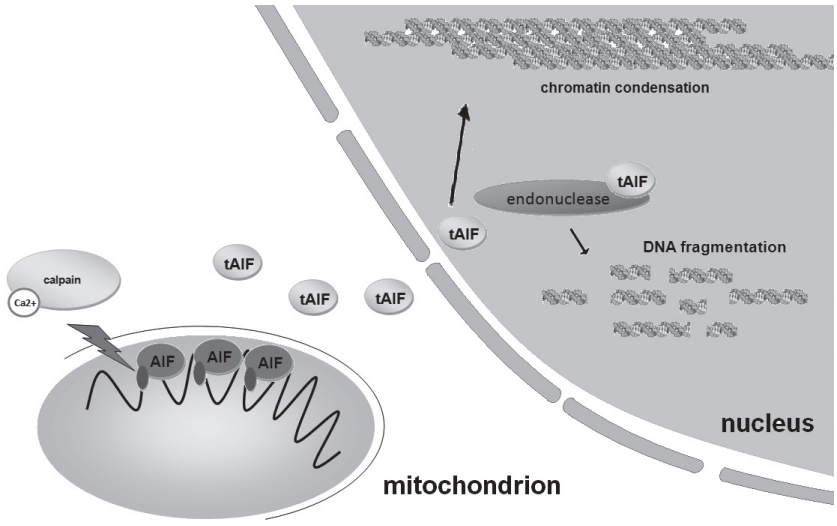

Figure 4. Calpain induces mitochondria-related apoptosis
Calpain-mediated limited proteolysis transforms AIF into tAIF (truncated form), thus releasing it from mitochondrial membrane anchor. Only the truncated AIF form may reach the nucleus and contribute to chromatin condensation and DNA fragmentation.

collagen fragments in a ruptured atherosclerotic plaque to reperfusion-induced neuronal injury after cerebral ischemia (Kobayashi *et al.*, 2002; von Wnuck Lipinski *et al.*, 2006; Rami *et al.*, 2007).

As calpains seem to be especially active in mitochondria-related cell death pathways, it was no surprise that their relationships with the mitochondrial apoptosis inducing factor (AIF) were searched for. AIF is a flavoprotein residing in the mitochondrial intermembrane space, strictly associated with the inner mitochondrial membrane but translocating to the cytosol and then to the nucleus in certain models of neurotoxicity, where subsequently it binds to DNA inducing its fragmentation, perhaps through recruitment of endonucleases (Susin *et al.*, 1999). AIF was demonstrated to contain a PEST motif (amino acids 529–562) near its carboxyl terminus, therefore — together with the interesting fact that AIF mediates almost specifically caspase-independent cell death — the suggestion of calpain involvement in its cleavage and activation was drawn. AIF seems practically immune to the typical factors influencing mitochondrial membrane permeabilization until calpain-mediated proteolysis into tAIF (truncated form) detaches it from a domain anchoring it to the membrane. Only then can the protein increase mitochondrial permeability and subsequently translocate to the nucleus (Fig. 4). Calpain-dependent AIF release seems to be a key element of an important signaling pathway that mediates neuronal cell death after cerebral ischemia (Polster *et al.*, 2005; Cao *et al.*, 2007).

Last but not least, there are strong suggestions of a possible protective role of calpains in TNFα-induced apoptosis in some cell systems (Lu *et al.*, 2002). Multiple investigations have indicated that TNFα may stimulate calpain-mediated IκB cleavage or phosphorylation thus inactivating it and increasing cellular levels of free NFκB transcription factor (Han *et al.*, 1999; Kouba *et al.*, 2001). That could in turn influence expression of a number of antiapoptotic genes resulting in saving a cell under certain conditions. Similar mechanism — including the NFκB — seems to be involved in ceramide/glutamate-triggered antiapoptotic pathway suggesting the existence of a special ceramide-calpain-NF-κB axis with prosurvival functions in certain conditions, especially in neurons. There, a rapid reduction of IκBα levels after calpain-mediated degradation of the protein results in nuclear translocation of NFκB p65 subunit and subsequent cell death evasion after several transcription modifications. Thus, NFκB activation by calpain may, up to a certain level, mediate the long-term effects of glutamate on neuron survival and memory formation (Schölzke *et al.*, 2003; Demarchi *et al.*, 2005).

CALPAINS INFLUENCE APOPTOSIS IN EXPERIMENTAL ONCOLOGY

If we assume that the basis of cancer development is a disturbed balance between cell proliferation and cell death, the interest oncologists find in calpains seems obvious. Studies in this field concern not only the details of cellular and molecular mechanisms of already existing anti-cancer therapies, but also increasingly the role of calpain in deregulation of apoptosis in neoplastic cells, the deciphering of which might be useful in designing new therapies and establishing the potential of calpains as prognostic or predictive factors. The starting point for any such considerations is the study of the actual amounts and activities of calpains in cancer cells compared to healthy ones.

Overexpression of calpains observed in a wide variety of human diseases gained special meaning in the field of haematological malignancies. Broad calpain invovlvement in significant apoptosis deregulation in those was postulated both *in vitro*, in leukemia/lymphoma cell lines, and *in vivo* or *ex vivo* observations. For example, calpain inhibitors were found to trigger apoptosis in human acute lymphoblastic leukemia (ALL) and non-Hodgkin's lymphoma (NHL) cells (Zhu & Uckun, 2000**)**. The cell lines studied in this work comprised Daudi (Burkitt's lymphoma cell line), Jurkat and MOLT lines and different B- and T-lineage ALL cell lines, most of them exhibiting relatively high calpain expression. Apoptosis induced by calpain inhibition was demonstrated in those to be mediated mostly through caspase-3; however, the results lacked the *in vivo* confirmation (Zhu *et al.*, 1999; Zhu & Uckun, 2000). The idea was further developed by Witkowski *et al.* (2002) in B-cell chronic lymphocytic leukemia (B-CLL), which is a relatively common leukemia type, characterized by a greatly decreased apoptosis rate rather than increased proliferation, making it especially valuable for studies of apoptotic mechanisms. These authors proved both overexpression and hyperactivity of μ-calpain specifically, the fact corresponding strongly with strikingly low caspase-3 activity observed in this disease. Moreover, they noted that inhibition of calpain activity caused a noticeable increase in spontaneous apoptosis rate in leukemic cells, thus suggesting a pivotal role of calpains in B-CLL apoptotic arrest. A special merit of those experiments lies in the use of *ex vivo* cells, allowing verification of the calpains' role under more native-like conditions than in alternative cell line studies.

Overexpression of calpains came into attention also in numerous solid tumors. Human colorectal adenocarcinoma cells were definitely proved to contain an increased,

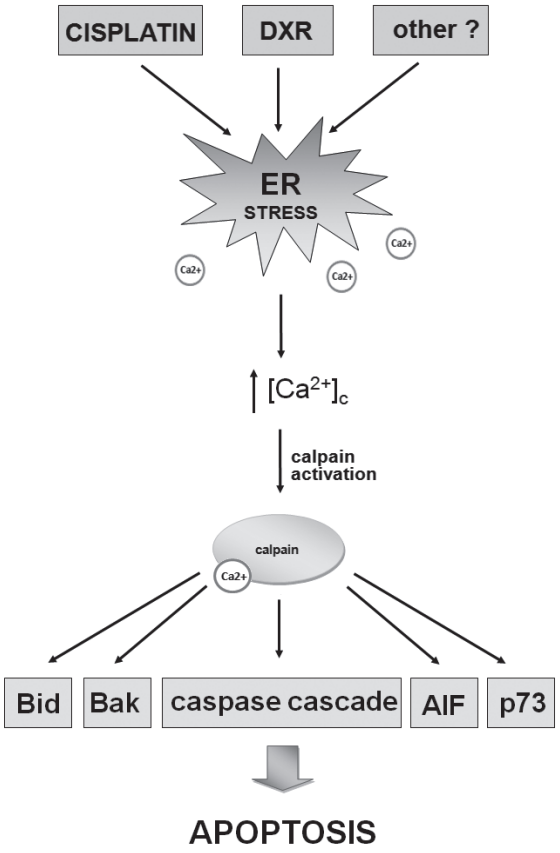

Figure 5. Chemotherapeutic agents act *via* calpain activation
Cisplatin, doxorubicin and possibly other anticancer drugs induce endoplasmic reticulum (ER) stress and increase intracellular calcium concentration leading to calpain activation. Activated calpains trigger numerous proapoptotic signaling pathways.

comparing to normal colonic mucosa, calpain amount, with an accompanying significant decrease of endogenous calpain inhibitors, especially calpastatin (Lakshmikuttyamma *et al.*, 2004). Other cancers with appreciated calpain involvement among their pathomechanisms are, e.g., prostate cancer (Zhu *et al.*, 1995; Mamoune *et al.*, 2003), lung adenocarcinoma (Liu *et al.*, 2008) or melanoma (Del Bello *et al.*, 2007).

Calpain overexpression was noted and correlated with increased activity of a number of proapoptotic factors in a set of highly malignant brain tumors including glioblastoma multiforme, anaplastic astrocytoma, neuroblastoma and ependymoma. In those tumors the significant increase in both amount and activity of calpain was accompanied by enhanced activities of Bax, caspase-3 and caspase-9 and resulting internucleosomal DNA fragmentation, suggesting that calpain function in them focuses strongly on regulating apoptotic events. Evidence was also gathered on calpain involvement in apoptosis of neuroblastoma cell lines after exposure to flavonoids, executed through the mitochondrial pathway. Functional studies performed in this field are rather scant and mostly performed on cell lines; however, the study designed by Ray and coworkers included also *ex vivo* experiments on tumor cells obtained from oncologically diagnosed patients (Ray *et al.*, 1999; 2002; Das *et al.*, 2006).

The calpain family seems also to constitute an important player in chemotherapy-related cell death. Calpain activation is supposed to be a major agent in the proapopototic action of cisplatin in human lung adenocarcinoma (Liu *et al.*, 2008); there are also some hints suggesting its possible similar role in human head and neck squamous carcinoma cells (Kim *et al.*, 2008). During experiments concerning human melanoma cells, cisplatin-induced calpain activation occurred early in apoptosis leading to the triggering of the intrinsic apoptotic pathway through cleavage of Bid protein (Mandic *et al.*, 2002), cisplatin led also to caspase-3 and caspase-7 activity stimulation, the process being preceded by calpain activation (Del Bello *et al.*, 2007). Similarly, in lung adenocarcinoma the data demonstrate that calpain-mediated cisplatin-induced apoptosis is exerted through activating Bid and AIF (Liu *et al.*, 2009) which then regulate the mitochondrial apoptotic pathway. Moreover, there are notions suggesting that the calpain-mediated pathway is the earliest one, actually dominating the cisplatin-induced cell death (Liu *et al.*, 2008). Different, albeit still calpain-controlled mechanism is postulated for cisplatin-mediated cell death of human ovarian carcinoma cell lines, where increased calpain activity leads to cleavage of p73 isoforms with subsequent proapoptoic shift in their intracellular ratio, the result being cell death mediated probably mostly by PUMA (p53-upregulated modulator of apoptosis) and NOXA (one of the Bcl-2-related proapoptotic proteins) (Al-Bahlani *et al.*, 2011).

The anthracyclin compound doxorubicin (DXR) is another well established antitumor agent, whose effect on tumor cells by potential association with calpain was already mentioned in the chapter concerning Bcl-2 family/calpain relations. Indeed, calpain inhibitors were also able to modulate DXR proapoptotic potential, mostly the interaction of the drug with the mitochondrial apoptotic pathway. As described earlier, calpain-mediated Bak cleavage was shown to stimulate cell death. DXR was also found to induce apoptosis through caspase-12 mediated pathway, the result being, among others, a broad spectrum of its side-effects during chemotherapy, including cardiomyocyte damage and fertility impairment. Both these effects seem to be due, at least partially, to calpain interactions with caspases (Jang *et al.*, 2004; Ben-Aharon *et al.*, 2008).

Connection of cisplatin and DXR effects with calpain system may be easily explained when one considers that both these chemotherapeutics are supposed to generate high levels of ER (endoplasmic reticulum) stress accompanied by intracellular release of calcium ions (Mandic *et al.*, 2003; Jang *et al.*, 2004; Linder *et al.*, 2005), and that increased calcium concentration is a crucial calpain-activating factor. Activated calpain in turn begins the whole cascade of events eventually leading to apoptosis (Fig. 5).

CALPAINS INFLUENCE NEURODEGERENATIVE CELL DEATH

The idea of the involvement of calpains in neuronal apoptotic response to various stimuli appeared first in animal brain ischemia models, evolving from simple observations of high intracellular calcium levels accompanying ischemia resulting in neuronal death (Yamashima *et al.*, 1996), then developing in time to the major theory of hypoxia-induced, ischemic neurodegeneration model, widely accepted for various forms of human nervous system impairment. Besides neuronal ischemia or brain injury, calpain overexpression was noted and — not surprisingly — causatively associated with neuronal death in a number of specific neuropathological processes, the

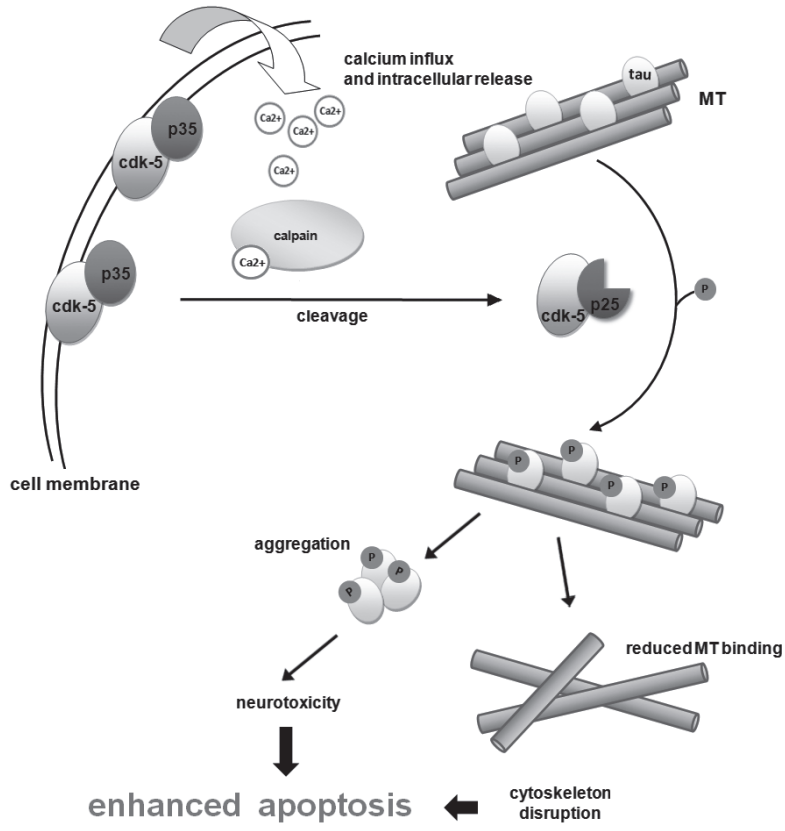

Figure 6. Activity of calpains in neurons may lead to neurodegeneration.
Calcium-activated calpains cleave p35 complexed with cdk-5 into p25, leading to hyperactivation and delocalisation of the kinase. p25/cdk-5 phosphorylates tau protein, thus decreasing its microtubule (MT)-binding potential. Phosphorylated tau aggregates creating neurotoxic, disease-specific neurofibrillary tangles. Microtubules deprived of the stabilizing effect of tau fail to maintain cytoskeleton integrity.

most prominent being Alzheimer's and Parkinson's diseases (Saito *et al.*, 1993; Mouatt-Prigent *et al.*, 1996). Progressive neuronal cell loss in the brains of Alzheimer's disease patients is supposed to result from extracellular β-amyloid peptide aggregates and intracellular accumulation and aggregation of hyperphosphorylated tau protein-composed neurofibrillary tangles. Calpains can affect the metabolism of both tau and amyloid precursor protein (APP). Tau protein counts among direct substrates of calpains and tau cleavage and phosphorylation are supposed to be the very first stages leading to neurotoxicity observed in Alzheimer's disease (Wang *et al.*, 2008). In addition, calpastatin overexpression seems to prevent pathological tau protein phosphorylation through restricting the abovementioned influence of calpain on cdk-5 activation; after calpain-mediated cleavage of p35 (neuron-specific activator of cdk-5) to p25, the p25/cdk-5 complex, detached from the cellular membrane, phosphorylates tau, decreasing its microtubule binding potential and therefore leading to deep cytoskeletal disruption and enhancement of cell death rate, the effect obtainable also after direct cdk-5 inhibition (Higuchi *et al.*, 2004, Zheng *et al.*, 2005) (Fig. 6).

Calpain inhibitors proved also to be helpful in suppressing APP-induced activation of neuronal caspase-3, thus limiting the range of neuronal cell death characteristic for Alzheimer's disease (Kuwako *et al.*, 2002); similar results were obtained in animal models of the disease (Vaisid *et al.*, 2007). As those observations may have obvious clinical implications and the struggle of pharmacology researchers against Alzheimer's disease is still ineffective, no wonder then that calpain inhibitors have begun lately to be noted as promising therapeutic agents (Di Rosa *et al.*, 2002).

Degeneration of dopaminergic neurons in the mid-brain (in substantia nigra specifically) is a key process in Parkinson's disease; however, some additional accompanying mechanisms are also suggested. In experimental parkinsonism models degeneration of spinal cord motoneurons was observed in the course of disease progress and calpains seem to be responsible for that (Samantaray *et al.*, 2006), the mechanism involving most probably an increase in the Bax:Bcl-2 ratio, release of cytochrome *c* from mitochondria and the whole intrinsic apoptosis machinery (Das *et al.*, 2005). Numerous studies in rodents and cell culture models of Parkinson disease suggest that treatment with calpain inhibitors (e.g., calpeptin) can prevent neuronal death and restore their functions, offering a chance of a novel Parkinson disease adjuvant therapy (Samantaray *et al.*, 2008).

Generally, in neurodegenerative disorders-related apoptosis calpain is supposed to work synergistically with caspase-3 in both apoptosis induction and execution through control over its regulatory proteins, e.g. XIAP, but also directly influencing executor caspases (Blomgren *et al.*, 2001; McCollum *et al.*, 2002; Neumar *et al.*, 2003); however, cell death or survival is strictly stimulus- and site-dependent. Thus, under certain conditions calpains may exert both neuroprotective and neurodegeneration-stimulating effects (Moore *et al.*, 2002). As DNA damage is thought to be a most crucial factor initiating clinically-relevant neuronal death in ischemic/reperfusion incidents (the simplest example being probably the stroke), known calpain control over p53 protein is properly assumed to be crucial in a scheme of neurodegenerative cell death (Sedarous *et al.*, 2003). Also, as p73 is involved in the survival of central nervous system neurons (Pozniak *et al.*, 2002), the potential role of calpains in modulating its activity can only give them a stronger place in the neural cells' apoptotic network; similarly, AIF mentioned earlier to be released from mitochondria by calpains, is a factor emerging vital during neuronal death following ischemic brain injury (Cao *et al.*, 2007).

Cathepsins, another family of cysteine proteases numbered along cell death co-regulators are supposedly crucial for neuronal apoptosis. Thus, the first concept of calpain–cathepsin–caspase interactions in neuronal cell death was raised by Yamashima who suggested that interactions between those three types of cysteine proteases are specific for neuronal death and crucial in executing it (Yamashima, 2000). The idea, however, still awaits further evaluation and development.

PERSPECTIVES

Numerous studies calling into question the actual potential of calpains to influence apoptotic processes relied mostly on the uncertain selectiveness of calpain inhibitors used in the research. At the same time, the postulated lack of specificity appeared to be the major limitation for potential use of calpain inhibitors as therapeutic agents. An argument to be put forward appeared at last in a series of recently published papers, dealing with a new series of highly selective calpain inhibitors (Donkor *et al.*, 2003; Guan *et al.*, 2006; Korukonda *et al.*, 2006) synthesized for a specific purpose of calpain function studies. They seem to influence apoptosis specifically, potently, time- and concentration-dependently, and (which appears to be a common and basic finding in calpain function studies) differently in different tissues and cell lines, supporting the concept that conventional calpains, however ubiquitous, may act in highly variable if not totally opposite ways.

Earlier observations of a special susceptibility of haematological malignancies to calpain-controlled apoptosis were confirmed in recent experiments on Daudi and Jurkat cell lines (Korukonda *et al.*, 2006). Apoptotic response in reaction to calpain inhibitors was demonstrated for various stages of the process, ranging from caspase activation to AnnexinV binding due to PS externalization and DNA internucleosomal fragmentation, thus enabling the investigators to monitor calpain influence over these stages. Those observations were especially reliable thanks to the novel, highly specific inhibitors used.

Besides oncology, immunology and neurobiology/neurology, other (sometimes rather incidental) observations of calpain-mediated apoptosis emerge quite often in very different clinical domains. Lately, for example, calpains' involvement in neutrophil apoptosis was rediscovered during studies on the effect of protease inhibitors in AIDS patients, where it was noted that one of the main results of the treatment not directly related to its antiviral activity, namely a gross reduction of neutrophil cell death, is due to cross-inhibition of calpains by those drugs (Lichtner *et al.*, 2006). It seems that unintentional therapeutic use of calpain inhibition is already a rather common situation.

There is probably still much to discover also in the field of miscellaneous intracellular interconnections of calpains with other signal proteins. New perspectives in calpain research are being opened with practically every new observation concerning cell death. For instance, calpains were lately found to induce apoptosis in pulmonary microvascular endothelial cells during sepsis (Hu *et al.*, 2009). Among the many different proteins supposed earlier to be specifically involved in endothelium death mechanisms are Hsp chaperones (Gawad *et al.*, 2009), already noted to influence calpains in various tissue systems (Sahara & Yamashima, 2010).

Calpains may eventually become first-class players in the understanding and then modulating cell death processes. Alongside with their established position in the basic science domain and growing appreciation for their clinical implications, noticeable boost in pharmacological chemistry industry efforts to manipulate calpain activities in a wide and diverse spectrum of diseases follows (Neffe&Abell, 2005; Shirasaki *et al.*, 2006a, 2006b, 2006c). Conventional calpains present an already established and potential target for anti-cancer drugs, their role in neurodegeneration is more and more appreciated and they still encroach over new fields of clinical expectations, including those of experimental cardiology, clinical immunology and virology, where they promise new understanding and applications.

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