A few days after a transient brain ischemia, the pyramidal neurons in the cornu Ammonis (CA) 1 sector of the hippocampus undergo selective death, a process named delayed neuronal death (DND). Cell death may occur as necrosis and/or apoptosis, and both have been reported to take place in DND. The cell’s decision between apoptosis and necrosis may depend on the strength of the insult, the balance of downstream signal transduction systems, and the expression level of pro- and anti-apoptotic or necrotic factors. Cytosolic calcium (Ca\(^{2+}\)) overload specifically occurs in the CA1 neurons after ischemia and thus is considered a common triggering event of the death cascade. As Ca\(^{2+}\) activates a wide array of intracellular enzymes, many Ca\(^{2+}\)-targeted enzymes have been implicated in DND. Among these, the present review will focus on the cysteine proteases calpain and cathepsins (B and L). In addition, their possible interactions with another family of cysteine proteases, caspases, will be discussed in relation to the cellular fate toward apoptosis or necrosis.

Key words: brain ischemia, calpain, cathepsin, delayed neuronal death.

INTRODUCTION

Cerebral ischemia has profound effects on the brain, inducing both immediate and delayed injuries. Even a transient ischemic insult is sufficient to cause selective damage to certain sensitive brain regions. The cornu Ammonis (CA) 1 sector of the hippocampus is considered such a region, since a transient global ischemia is able to cause neuronal death a few days after cerebral ischemia. This phenomenon is designated as delayed neuronal death (DND) and was first described in gerbils in 1982.\(^1,2\) Later, its clinical relevance was also confirmed.\(^3,4\) Both necrosis\(^1-5\) and apoptosis have been reported as a type of cell death of DND, but its molecular mechanisms remain largely unclear.\(^6,7\)

The role of calcium (Ca\(^{2+}\)) in ischemic neuronal death is well recognized.\(^8\) An excessive postischemic Ca\(^{2+}\) mobilization occurs in CA1 neurons\(^9,10\) by both glutamate-induced Ca\(^{2+}\) influx\(^11,12\) and/or inositol 1,4,5-trisphosphate (IP\(_3\))-induced Ca\(^{2+}\) release from intracellular stores.\(^13,14\) Elevated intracellular Ca\(^{2+}\) concentration in turn activates a series of enzymes including proteases, phospholipases, phosphatases and protein kinases. It may also trigger the synthesis of the free radical nitric oxide (NO), involved in neuronal apoptosis.\(^15\)

Among the Ca\(^{2+}\)-activated enzymes, the authors will focus on the cysteine proteases calpain and cathepsins (B and L). In addition, their possible interactions with another family of cysteine proteases, caspases, will be discussed in relation to the cellular fate toward apoptosis or necrosis.
Cysteine proteases in neuronal death

357
generally requires an acidic pH. Thus, they are localized
predominantly to the lysosomes (cathepsins B, H, L, S, C
and K), but also to the nucleus (cathepsins B and L) and
cytosol (cathepsins B and E). Most cathepsins have
molecular weight (MW) values of 20–35 kDa (with the
exception of cathepsin C, which is approximately 200 kDa).
Unlike calpains, they lack Ca\(^{2+}\)-binding domains.
Numerous cathepsin inhibitors exist, the most abundant of
which are the cystatins. The latter interact with active
proteases to bind them tightly and essentially irreversibly.
Their function is to inhibit cathepsins that escape
compartmentalization and protect cells, tissues and the
circulation from unwarranted cysteine protease activity.

**SUBSTRATES OF CALPAIN AND CATHEPSIN**

The ubiquitous and constitutive expression of \(\mu\)-
and \(m\)-calpains makes it difficult to precisely define their
physiological functions. A large number of calpain sub-
strates have been demonstrated, mainly in vitro, including
cytoskeletal proteins, growth factor receptors, and
transcription- and cell cycle-related proteins (Table 1).
In relation to the latter, selective nuclear transport of
\(\mu\)-calpain has been demonstrated.

It is worth noting that calpastatin itself is also a target
of calpain, and \(\mu\)-calpain is capable of activating
\(m\)-calpain. Thus, a calpain cascade may coordinate the
functions of calpains in cells. A variety of calpain functions
have been proposed including intracellular protein de-
gradation, platelet aggregation, erythrocyte aging, neu-
trophil activation as well as long-term potentiation in the
hippocampus. Detailed data on the physiological functions
of calpains are available in other reviews.

Cathepsins were shown to participate in the extracel-

lular matrix remodeling, and cathepsin K is considered the
most potent elastolytic enzyme known. Collagen cleavage
by cathepsin B and fibronectin degradation by cathepsin
D have been demonstrated. Several cytoskeletal proteins
are substrates of cathepsin D including myelin basic
protein, neurofilament proteins, microtubule-associated
protein 2, tubulin and \(\beta\)-amyloid protein. Cathepsins
are essential for the generation of antigenic peptides in the
major histocompatibility complex class II antigen presenta-
tion (cathepsin S) as well as for the processing of prohor-
mone molecules, such as pro-endothelin-1 (cathepsin D) and
prorenin (cathepsin B). Cathepsin B is also capable of
cleaving the myristoylated alanine-rich C kinase sub-
strate (MARCKS), a major cellular substrate of protein
kinase C.

Cathepsin L has been implicated in tumor progression
and bone resorption. Interestingly, members of the
caspase (the other major cysteine protease) superfamily,

**Table 1** A list of selected calpain substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoskeletal proteins</td>
<td></td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>20</td>
</tr>
<tr>
<td>Actin</td>
<td>23</td>
</tr>
<tr>
<td>Spectrin</td>
<td>24, 25</td>
</tr>
<tr>
<td>NF-68</td>
<td>26</td>
</tr>
<tr>
<td>Tubulin</td>
<td>20</td>
</tr>
<tr>
<td>Tau</td>
<td>27</td>
</tr>
<tr>
<td>Ca(^{2+})-binding proteins</td>
<td></td>
</tr>
<tr>
<td>Caldesmon</td>
<td>34</td>
</tr>
<tr>
<td>Calponin</td>
<td>34</td>
</tr>
<tr>
<td>Enzymes</td>
<td></td>
</tr>
<tr>
<td>Calpastatin</td>
<td>35</td>
</tr>
<tr>
<td>Nitric oxide synthase</td>
<td>36</td>
</tr>
<tr>
<td>PKC</td>
<td>20</td>
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<tr>
<td>Calcineurin</td>
<td>20</td>
</tr>
<tr>
<td>Growth factor/cytokine receptors</td>
<td></td>
</tr>
<tr>
<td>EGF receptor</td>
<td>28</td>
</tr>
<tr>
<td>PDGF receptor</td>
<td>20</td>
</tr>
<tr>
<td>Transcription and translation factors</td>
<td></td>
</tr>
<tr>
<td>AP-1 (c-Fos/c-Jun)</td>
<td>29</td>
</tr>
<tr>
<td>c-Myc</td>
<td>29</td>
</tr>
<tr>
<td>CREB</td>
<td>29</td>
</tr>
<tr>
<td>eIF-4E</td>
<td>30</td>
</tr>
<tr>
<td>Cell cycle/tumor suppressor proteins</td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>31</td>
</tr>
<tr>
<td>p53</td>
<td>32</td>
</tr>
<tr>
<td>NF2 (merlin, schwannomin)</td>
<td>33</td>
</tr>
</tbody>
</table>

NF-68, neurofilament 68 kDa protein; PKC, protein kinase C; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; AP-1, activator protein-1; CREB, cyclic AMP response element-binding protein; eIF-4E, eukaryotic initiation factor-4E; c-Myc, c-myelocytoma oncogene product.

**Fig. 1** Time course of [Ca\(^{2+}\)], elevation in the monkey hippocampal slice during hypoxia–hypoglycemia (adapted from Fig. 7a of Reference 16). The largest [Ca\(^{2+}\)], elevation demonstrated by relative fluorescence intensity was observed preferentially in the CA1 sector (\(\triangle\)), in contrast to the [Ca\(^{2+}\)], elevation in the CA3, CA4 and the dentate gyrus (a summing graph shown by (\(\bigcirc\))).
namely caspase-11 and to a lesser extent caspase-1, have recently been shown to be substrates of cathepsin B, while caspase-3 has been shown to be a substrate of cathepsin L. These data, together with the observation of caspase-mediated calpastatin degradation, suggest a potential interaction between the caspase and papain superfamilies in integrating their cellular effects.

CALPAIN AND CATHEPSIN INVOLVEMENT IN ISCHEMIC DELAYED NEURONAL DEATH

Analyzing postischemic neuronal Ca\(^{2+}\) mobilization by an in vitro ischemia experiment using acute hippocampal slices, Yamashima et al. measured intracellular free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) during hypoxia–hypoglycemia. The area of the maximum [Ca\(^{2+}\)]\(_i\) elevation in the slice (Fig. 1) was selectively localized to the CA1 sector, the area of DND in vivo. In previous studies, indirect evidence of Ca\(^{2+}\)-induced calpain involvement in DND has been demonstrated by the finding of calpain-mediated fodrin degradation, associated with Ca\(^{2+}\)-induced calpain activation in CA1 neurons after brain ischemia. Using an antibody that specifically reacts with an activated form of \(\mu\)-calpain and a primate model of 20-min whole-brain complete ischemia, Yamashima et al. provided direct evidence of calpain activation prior to DND. At immunohistochemistry, the perikarya of CA1 neurons, immediately after the ischemic insult, showed a specific staining of activated \(\mu\)-calpain (Fig. 2a, b) as compared with both the controls and the

![Calpain in ischemic hippocampus](image)

**Fig. 2** Calpain in ischemic hippocampus. (a, b) Immunohistochemistry using anti-activated \(\mu\)-calpain antibody. A significant increase in immunoreactivity is observed in ischemic CA1 neurons (b) compared with controls (a) showing no staining (×700; bar = 10\(\mu\)m). (c) Western blot analysis of control (C) and postischemic (I) hippocampal sectors using antibodies against inactive (pre-\(\mu\), upper half) or activated (post-\(\mu\), lower half) \(\mu\)-calpain. Proteins extracted from the CA1, CA2/3 and CA4/DG (dentate gyrus), respectively, are presented from left to right. The 80 kDa (inactive) \(\mu\)-calpain (pre-\(\mu\)) is significantly decreased in the ischemic CA1 sector compared with the control. In contrast, 76 kDa (activated) \(\mu\)-calpain (post-\(\mu\)) showed a significant increase, specifically in the postischemic CA1. (d) Immunoelectron micrographs of CA1 neurons immediately after 20 min hypoxia–hypoglycemia, showing that activated \(\mu\)-calpain is localized at the vacuolated membrane of lysosomes (arrows) (×11 500; bar = 1 \(\mu\)m).
remaining ischemia-resistant hippocampal neurons (data not shown). This observation was confirmed by western blot analysis of the respective hippocampal sectors (Fig. 2c). Most importantly, the \([\text{Ca}^{2+}]_i\) levels increased only transiently during hypoxia–hypoglycemia \textit{in vitro} (Fig. 1), while calpain activation \textit{in vivo} persisted long after \([\text{Ca}^{2+}]_i\) had returned to the normal level. Immunoelectron microscopy of the hippocampal slices using anti-activated \(\mu\)-calpain antibody revealed that activated \(\mu\)-calpain was localized at the vacuolated or disrupted membrane of lysosomes (Fig. 2d). Taken together, these data suggest that excessive calpain activation in postischemic CA1

![Immunohistochemistry for cathepsin B in CA1 (a,b,e) and CA3 (c,d) neurons. CA1 neurons 1 h after the ischemic insult (b) show a presence of extralysosomal cathepsin B immunoreactivity in contrast to the ischemia-resistant CA3 neurons (d). After CA-074 treatment, cathepsin B immunoreactivity in the CA1 neurons (e) is weak and comparable with the control (a). C, control CA3 neurons (a–d, \(\times 690\); e, \(\times 660\), bar = 20 \(\mu\)m).](image-url)
neurons may cause lysosomal membrane disruption with the resultant release of lysosomal enzymes triggering the cascade of DND.

Neuronal lysosomes are known to contain both calpains B and L, and the enzyme activity of both is increased in the CA1 sector 3–5 days after ischemia. Therefore, Yamashima et al. and Tsuchiya et al. aimed to identify the contribution of cathepsins B and L to ischemic CA1 DND. Immediately after ischemia, the cathepsin B immunoreactivity of CA1 neurons was significantly increased, and was localized not only to the lysosomes but also to the perikarya or the neuropil (Fig. 3a,b). In contrast, the cathepsin B immunoreactivity in the ischemia-resistant hippocampal neurons also increased after ischemia but did not show extralysosomal distribution (Fig. 3c,d). Such a difference in the immunolocalization of cathepsin B was also evident at the ultrastructural level. When the cathepsin B-selective inhibitor CA-074 was applied to the monkeys immediately after the ischemic insult, the immunoreactivity of cathepsin B in the lysosomal granules of CA1 neurons became much weaker, and no extralysosomal immunoreactivity was observed (Fig. 3e).

Quantification of cathepsin B enzyme activity in each hippocampal sector displayed a similar pattern showing an increase in all sectors 3–5 days after the ischemic insult, the application of CA-074 diminishing this effect (Fig. 4a). Cathepsin L activity showed a similar pattern (Fig. 4b).

Therefore, Ca\(^{2+}\)-induced calpain overactivation after transient brain ischemia may lead to disruption of lysosomes with the resultant release of cathepsins. The latter may gradually degrade cell constituents, thus contributing to the development of DND (calpain–cathepsin hypothesis). The extralysosomal release of cathepsin enzymes occurring specifically in the CA1 sector may explain the restricted localization of DND to this hippocampal region.

**POTENTIAL INTERACTIONS OF CALPAIN, CATHEPSIN AND CASPASES IN DELAYED NEURONAL DEATH**

Recently, apoptosis (programmed cell death) has been proposed as a mechanism of neuronal damage in DND, particularly in rodents. Dying ischemic neurons in different brain regions, including CA1, showed morphological features of apoptosis and a positive labeling of nuclear double-strand DNA breaks in situ (terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay) together with DNA fragmentation. Since another superfamily of cysteine proteases, the aspartyl-specific cysteine proteases (caspases), is well known to have an important role in neuronal apoptosis during development and also in various pathological conditions, their involvement in ischemic DND is currently a major subject of investigation. Based on their regulation and sequence of activation, caspases are classified as initiator (e.g. caspase-8, -9, -10) and effector (e.g. caspase-3, -6, -7) classes. In rodents, caspase-3 activation in transient global ischemia has been reported as

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**Fig. 4** Enzymatic activities of cathepsins B and L in monkey hippocampal sectors as measured by fluorescence spectrophotometry of 7-amino-4-methylcoumarin (AMC) release (see Reference 54 for more details). ( ) CA1; ( ) CA2; ( ) CA3; ( ) DG + CA4. The residual cathepsin activity in the CA1 sector at postischemic day 5 after CA-074 (a selective cathepsin B inhibitor) treatment was 36% for cathepsin B (a) and 80% for cathepsin L (b), compared with the respective untreated day 5 groups. However, after E-64c (a non-selective inhibitor) treatment, the residual cathepsin activity in the CA1 sector was 14% for cathepsin B (a) and 43% for cathepsin L (b), compared with the respective untreated day 5 groups, 5d, untreated day 5 group; 5d(CA-074), CA-074-treated group; 5d(E-64c), E-64c-treated group. (a) \(*P < 0.01\) versus control; \(**P < 0.05\) versus control and day 5; \(***P < 0.01\) versus day 5. (b) \(*P < 0.05\) versus control; \(\neq\)not significant; \(**P < 0.01\) versus day 5.
showing a selective and prolonged caspase-3 mRNA and protein induction in the CA1 neurons prior to apoptosis.\textsuperscript{59-61} Furthermore, a specific inhibitor of caspase-3 significantly reduced CA1 neuronal death.\textsuperscript{59,61} Therefore, up-regulation of caspase-3 in dying CA1 neurons may be an important step in the development of the postischemic DND of rodents.

As mentioned, caspase-3 and calpain have common substrates in neurons, including $\alpha$-spectrin\textsuperscript{25} and neurofilament proteins.\textsuperscript{26} A temporal relationship between calpain/caspase-3 activation and $\alpha$-spectrin degradation prior to DNA fragmentation and apoptosis was established.\textsuperscript{62} Calpain activation in apoptosis upstream of caspases,\textsuperscript{63} and caspase-3-mediated calpastatin degradation\textsuperscript{35} inducing calpain activation in non-neuronal cells, were shown. Furthermore, cathepsin B activates caspase-11 upstream of caspase-3-mediated liver cell apoptosis,\textsuperscript{49} and cathepsin L activates caspase-3-like protease, also in liver cells.\textsuperscript{49} These data suggest that a mutual communication may exist between members of the two large cysteine protease superfamilies in executing their roles in DND (Fig. 5). Thus, a calpain–cathepsin–caspase cascade might be involved in the primate postischemic CA1 DND.

The role of mitochondria in necrosis has been recognized for decades, while their implication in apoptosis has recently also become clear.\textsuperscript{64} The induction of mitochondrial permeability transition (MPT), a process of disruption of mitochondrial membrane integrity, is considered a key step in the execution of apoptosis.\textsuperscript{65} Mitochondrial permeability transition is thought to be caused by factors such as mitochondrial calcium overload and results in release of cytochrome c and caspase-9 from the intermembrane space of mitochondria, which triggers the caspase cascade prior to apoptosis.\textsuperscript{64} In postischemic hippocampal slices, cytochrome c was released into the cytosol\textsuperscript{66} and the activation of MPT among various brain regions correlated with their susceptibility to ischemic damage.\textsuperscript{67} In addition, the MPT inhibitor cyclosporin A ameliorated hippocampal CA1 neuronal damage after transient global ischemia in rodents.\textsuperscript{68} Thus, it is tempting to speculate that in the primate postischemic DND, both calpain–cathepsin- and MPT-triggered caspase activation might induce neuronal degeneration and death (Fig. 5).

**Therapeutic Strategies and Future Perspectives**

Assuming that cysteine proteases are involved in the development of ischemic brain injury, it is reasonable to predict that their inhibition, even if applied after the ischemic insult, could have a protective effect on the degenerating postischemic neurons.\textsuperscript{69} In particular, calpain\textsuperscript{70} and caspase\textsuperscript{71} inhibition has successfully blocked ischemic CA1 DND in rodents. As described, selective cathepsin B
inhibition was able to prevent ischemic DND in monkeys.\textsuperscript{17} Also, the non-selective cysteine protease inhibitor E-64c that inhibits not only cathepsins B, H and L, but also calpain had a greater potency in reversing DND compared with the selective cathepsin B blocker (Fig. 4b).\textsuperscript{18} This is further evidence implicating calpain and cathepsin enzymes in the development of DND.

In addition to inhibitors of cysteine proteases and cyclosporin A, several drugs, such as the gamma-aminobutyric acid (GABA) agonist diazepam,\textsuperscript{72} the microtubule-disrupting agent colchicine\textsuperscript{73} and the immunosuppressant FK-506,\textsuperscript{74} displayed a neuroprotective effect on CA1 neurons after transient global ischemia. A similar effect was confirmed also for acidic fibroblast growth factor,\textsuperscript{75} hepatocyte growth factor,\textsuperscript{76} and interleukin-3.\textsuperscript{77} The effect of diazepam is probably mediated via the mitochondrial benzodiazepine receptor,\textsuperscript{64} in a similar way to cyclosporin A. Both cyclosporin A and FK-506 inhibit the cytoplasmic protein phosphatase calcineurin; however, FK-506 lacks the ability of inhibiting MPT. Therefore, calcineurin, a calpain substrate,\textsuperscript{20} may be involved in postischemic DND in a MPT-independent fashion.

It is now recognized that cell death may take place by necrosis or apoptosis. The prevalence of either type of death in various neuropathologies including ischemia is debatable at present.\textsuperscript{78,79} In particular, both necrotic and apoptotic features have been reported in CA1 DND. In various models of total brain ischemia, including the primate model described above,\textsuperscript{16–18} the features of CA1 neuronal death were essentially necrotic. However, evidence of apoptosis has also been reported in gerbils.\textsuperscript{6}

In the context of the involvement of calpain-cathepsin cascade in CA1 neuronal necrosis and of mitochondrial factors–caspase cascade in apoptosis, we would like to propose two possible models of the occurrence and prevalence of necrosis and apoptosis in CA1 DND (Fig. 6).

In both models an interaction between the two systems, calpain–cathepsin and mitochondrial factors–caspase, takes place prior to cell death. In one model, the two systems may separately lead to necrosis or apoptosis depending on the severity of the insult (Fig. 6a), and the cell fate is determined by the relative prevalence of their components. In the other model, a common calpain–cathepsin–caspase cascade (with the participation of mitochondrial factors) may execute cell death (Fig. 6b). That is, a cathepsin-mediated apoptosis\textsuperscript{40,49} and a caspase-mediated necrosis\textsuperscript{80–82} may simultaneously take place, and it is the severity of insult that determines whether cell death will be restricted to apoptosis only or whether necrosis will eventually follow. Future studies on the temporal and quantitative pattern of the gene expression of various pro- and anti-apoptotic and necrotic factors will shed more light on their precise role in DND as well as the contribution of apoptosis and necrosis to the development type of neuronal damage.

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