Copy edited on disc DNE/940/hol

Received: September 30, 1999

Accepted: December 8, 1999

Role of Mitochondria in Neuronal Apoptosis

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Abstract

Apoptosis is a controlled form of cell death that participates in the demise of neuronal cells during development, neurodegenerative disorders and exposure to neurotoxic agents. In recent years, the mitochondria have emerged as being pivotal in controlling apoptosis. They house a number of apoptogenic molecules that are released into the cytoplasm at the onset of apoptosis. These include cytochrome c, apoptosis-inducing factor and various caspases. Mitochondria also play an important role in intracellular Ca^{2+} regulation, which is crucial to excitotoxic neurodegeneration. Alterations in energy (ATP) production by mitochondria (due to hypoxia or mutations in genes encoding mitochondrial proteins of the electron transport chain) can induce apoptosis in neurons or increase their sensitivity to apoptosis.

Key Words

Apoptosis

Caspase

Cytochrome c

Hypoxia

Mitochondria

Introduction

In this paper, we will discuss the role of the mitochondria in neuronal apoptosis, giving examples from the literature and presenting some of our recent data on the induction of apoptosis by hypoxia. Apoptosis is an active mode of death, which was originally defined morphologically by the characteristic shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation of cells dying by this process. The biochemical pathways to apoptosis are rapidly being uncovered, revealing that it is a highly regulated process. This is in contrast to necrosis, which is typified by cell swelling and uncontrolled lysis. It is well established that apoptosis can be induced in neurons in in vitro culture systems. During recent years, there has been increasing evidence that apoptosis is also important for neuronal cell death in vivo, e.g. as a result of toxic insults, in neurodegenerative diseases and during development of the central nervous system. Many neurodegenerative disorders display evidence of mitochondrial dysfunction, which is interesting given the important role that the mitochondria are now known to play in apoptosis. In fact, mitochondrial changes have at times been considered as essential for apoptosis [1]. More recently, however, our group has proposed that apoptosis should be defined in terms of both morphology and biochemistry, notably the activation of a unique family of apoptotic proteases, the caspases [2].

Caspases

Mammalian caspases are a family of at least 14 cysteine proteases that are largely responsible for the ordered degradation of the cell that occurs during apoptosis. Their activation is one of the early steps committing a cell to this mode of cell death. They specifically cleave after Asp residues [3] and a comprehensive list of their many and varied substrates has recently been published [4]. The list includes proteins such as poly(ADP-ribose) polymerase, fodrin, nuclear lamins, huntingtin, presenilins, atrophin 1 and ataxin 3 [4].

Using a combinatorial approach the substrate specificities of the caspases have revealed three major groups [5] which have different preferences for the amino acid residues at positions P2--4 (Asp is always in the P1 position). Group 1 is comprised of caspases 1, 4 and 5 (i.e. those which predominantly play a role in the inflammatory response of the immune system and cytokine production). Group 2 caspases, which includes caspases 2, 3 and 7, have a preference for DEXD, while

group 3 caspases includes caspases 6, 8 and 9, all of which prefer the sequence IEXD. Analysis of the cleavage sites of proteins degraded by caspases during apoptosis suggests that group 2 caspases are mainly responsible for these events, and thus, they are often referred to as executioner caspases. On the other hand, group 3 caspases have been designated as apoptotic initiator caspases since they act upstream of group 2, directly cleaving and activating them [6].

The expression of caspases can be regulated in the brain. For example, the murine homologue of human caspase 2, Nedd 2 (neuronally expressed developmentally down-regulated) is expressed at high levels in the embryonic mouse brain but not in the adult [7]. This pattern of expression temporally correlates with massive developmental cell death in the nervous system. Moreover, caspase-3- and caspase-9-deficient mice display marked alterations in brain structures that mainly result from decreased neuronal apoptosis [8--10]. Caspase protein expression can also be regulated in response to neuronal injury, particularly due to ischemia [11, for review].

Mechanisms of Caspase Activation

Caspases are normally present in the cell in an inactive proform that requires proteolytic processing to generate the heterodimeric active enzyme. Therefore, activation of group 3 initiator caspases is a critical event in the induction of apoptosis. This can occur via different mechanisms [6, 11], which may involve cleavage by other proteases, the ligation of plasma membrane receptors, or a pathway involving the mitochondria. Firstly, caspases can be activated by the proteolytic activity of other caspases (previously activated), by granzyme B [12], calpain [13] or lysosomal cathepsin [14]. Secondly, ligand binding to members of the TNF receptor/NGF receptor family, which includes Fas (APO-1, CD95) and the p55 TNF receptor, can induce group 3 caspase activation via adaptor molecules. Of more interest to the nervous system (where Fas is not normally expressed) is the neurotrophin receptor $p75^{NTR}$, which is also a member of the TNF receptor superfamily [15]. However, apoptosis was shown to develop only in certain cases of ligand binding to $p75^{NTR}$, while in some other cases apoptosis is triggered in the absence of ligand binding [16, for review].

Finally, the mitochondrial pathway to caspase activation involves the cytoplasmic formation of the ‘apoptosome’ complex [17, 18], which contains the mitochondrial intermembrane space protein cytochrome c and the cytoplasmic proteins Apaf-1 [18]

and procaspase 9 [17] (fig. 1). Formation of the apoptosome induces the processing of procaspase 9 to the active enzyme. The substrate specificity of caspase 9 suggests that it may then cleave procaspases 3 and 7 and thus initiate the proteolytic cascade essential to apoptosis. It has been postulated that cytochrome c binds to the WD repeats of Apaf-1 in an ATP (or dATP)-dependent fashion, inducing oligomerization and conformational changes that allow Apaf-1 to bind to procaspase-9 [17, 19, 20]. Thus, it is assumed that Apaf-1 lies dormant in the cytoplasm until cytochrome c is available to activate it. Since cytochrome c normally resides within the intermembrane space of the mitochondria, it is necessary that it be released by some trigger.

Release of Mitochondrial Proteins to Trigger Apoptosis

There are two competing theories to explain how cytochrome c escapes from the mitochondria to the cytoplasm during the early phase of apoptosis [21]. In the first, there is swelling of the mitochondria and rupture of the outer membrane, allowing intermembrane space proteins, including cytochrome c, to escape. This has been proposed to occur either with [22] or without [23] prior mitochondrial permeability transition (MPT). MPT involves opening of a ‘megachannel’ called the permeability transition pore [1, 24] which consists of several proteins (including the voltage dependent anion channel, VDAC, the adenine nucleotide transporter, ANT, and cyclophilin D) that are located in both the inner and outer mitochondrial membranes [25]. In experiments with isolated mitochondria, opening of the pore allows water and solutes to enter, thereby inducing mitochondrial swelling.

However, in many types of apoptosis, the mitochondria remain morphologically normal. So other mechanisms might exist. The second model predicts the formation of a channel in the mitochondrial outer membrane that is large enough to allow cytochrome c to pass through. It has recently been shown that certain proapoptotic members of the Bcl-2 family (Bax and Bak) can interact with VDAC (also called mitochondrial porin) and accelerate its opening to allow the passage of cytochrome c [26]. In contrast, the antiapoptotic protein Bcl-x_L prevents cytochrome c release through the channel. Bid can induce Bax to undergo a conformational change resulting in cytochrome c release, presumably via a channel formed by either Bax or a Bid/Bax complex [27].

Apart from cytochrome c, the intermembrane space of mitochondria also contains a number of other proteins that are involved in apoptosis, e.g. AIF and various procaspases. During apoptosis, AIF is released from the mitochondria and translocates to the nucleus, where it causes chromatin condensation and high-molecular-weight fragmentation of the DNA [28]. AIF has also been reported to induce purified mitochondria to release cytochrome c [28]. Its role in neuronal apoptosis has not yet been determined.

A number of procaspases have been shown to have a mitochondrial localization including procaspase 3 [29--33], 2 [33, 34], 7 [33] and 9 [31, 33--35]. It has been shown that these caspases are released from the mitochondria during apoptosis to the cytoplasm, where they contribute to the amplification of the caspase cascade [29, 31--33, 35]. Of particular interest to neuronal cells is the finding that procaspase 9 is localized in the mitochondria of many neurons, and that during ischemic brain injury, caspase 9 translocates to the nucleus [35]. Apoptosis-inducing agents that induce cytochrome c release in PC12 cells cause a concomitant release of caspase 9 [35]. There is a similar release of caspase 9 from mitochondria in ischemia-damaged neurons [35]. Since neurons are nonproliferative cells, it makes sense that they might sequester caspase 9 in the mitochondria (away from Apaf-1) in order to protect against accidental death.

Bcl-2 Family as Regulators of Apoptosis

A number of proteins that regulate apoptosis are known to localize to the mitochondria. In particular, members of the Bcl-2 family of apoptotic regulators are localized to the mitochondrial outer membrane. Some family members, including Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1 function to inhibit apoptosis, whereas other members such as Bax, Bak, Bcl-x_S, Bad, Bid, Bik and Bim are proapoptotic [36, for review]. Their most frequently reported mode of action is regulation of cytochrome c release from the intermembrane space. However, cells overexpressing Bcl-2 are also protected from the induction of apoptosis caused by injection of cytochrome c into the cytoplasm [37, 38]. Similarly, microinjection of cytochrome c does not overcome the antiapoptotic effects of Bcl-x_L [39]. Shimizu et al. [26] have recently shown in liposomes that proapoptotic Bax and Bak can interact with the VDAC [26] to allow the passage of cytochrome c, while antiapoptotic Bcl-x_L prevents this permeation of cytochrome c through the VDAC. Bax has also been shown to interact with the ANT,

which forms part of the mitochondrial permeability transition pore [40]. Since permeability transition pore inhibitors such as cyclosporin A and bongkrekic acid can inhibit Bax/Bak-induced cytochrome c release in isolated mitochondria [41, 42], it is probable that the interaction of Bax and Bcl-x_L with components of the permeability transition pore (including VDAC and ANT) is involved in their regulation of apoptosis.

It should be noted that members of the Bcl-2 family are multifunctional, and regulation of cytochrome c is only part of their activity. Bcl-x_L, Bcl-2 and Bax have been reported to have ion channel activity in their own right, although it is as yet unclear whether the ion channel activity has a physiological role [43, 44]. Bcl-2 family members are also localized to other intracellular membranes, apart from mitochondria such as the endoplasmic reticulum and nuclear membranes [45--47]. The significance of this is unknown, although Bcl-2 has been shown to regulate endoplasmic reticulum-associated Ca^{2+} fluxes [48].

The antiapoptotic proteins Bcl-2 and Bcl-x_L have been found to interact with a variety of cellular proteins, including the protein kinase Raf-1, the protein phosphatase calcineurin, the GTPases R-Ras and H-Ras and the p53-binding protein p53-BP2 [49], whereas Bax does not interact with these proteins. It has also been reported that Bcl-x_L binds to cytochrome c, which suggests that it may have a quenching effect on any leakage from the mitochondria [50]. The familial Alzheimer's disease gene products, presenilin-1 and presenilin-2, have been found to interact with Bcl-x_L [51], and although mutant presenilins themselves do not induce apoptosis, they are thought to sensitize cells to apoptosis by diverse stimuli including trophic factor withdrawal and amyloid β-peptide [52, 53]. Presenilins could modulate sensitivity to cell death by regulating Bcl-x_L activity.

Mitochondrial Control of Life and Death (apart from via Release of Apoptogenic Proteins)

Mitochondria have long been dubbed the 'powerhouse of the cell', providing cells with the majority of their ATP. ATP synthesis is coupled to electron transfer through the formation of the H^+ electrochemical gradient across the inner mitochondrial membrane. Cytochrome c functions as part of this chain to shuttle electrons from complex III to complex IV. The loss of cytochrome c from mitochondria can therefore result in disruption of electron transport and ATP production. However, although a drop in intracellular ATP level has been observed during apoptosis, it often occurs

relatively late in the process [54], perhaps because only a proportion of the total cytochrome c is released, or because the glycolytic pathway serves to maintain ATP production during this time. This is consistent with the view that energy levels are critical in determining the mode of cell death, i.e. apoptosis or necrosis [55, 56]. In cultures of cerebellar granule cells, it has been shown that glutamate can induce either early necrosis or delayed apoptosis, depending on the mitochondrial potential and energy levels, suggesting that mitochondrial function is a critical factor that determines the mode of neuronal death in excitotoxicity [57]. Moreover, depletion of ATP from nonneuronal cells causes them to undergo necrosis in the face of a toxic insult rather than apoptosis when ATP levels are high [58].

Mitochondria are the major source of superoxide anion production in cells due to leakage of electrons from the respiratory chain. Superoxide production is increased during apoptosis induced by widely different stimuli [59, 60]. In fact, oxidative stress has been proposed as a mediator of apoptosis [61] and has long been associated with ageing and neurodegenerative diseases [62, 63]. It has recently been shown, however, that mitochondrial generation of superoxide in apoptosis is triggered by the loss of cytochrome c, suggesting that oxidative stress (from a mitochondrial source) is a consequence rather than an inducer of the apoptotic process [60]. However, in many instances it has been demonstrated that oxidative stress can directly induce apoptosis [64, 65], and in neuronal cells, oxidative stress has been shown to induce a form of death with characteristics of both apoptosis and necrosis [66]. It is possible that the induction of oxidative stress in neuronal cells as a result of superoxide production has an amplifying effect on the neurodegeneration seen.

Another important function of mitochondria is the sequestration of Ca^{2+} into the matrix via a Ca^{2+}-permeable uniporter in the inner mitochondrial membrane [67, 68]. A rapid and prolonged entry of Ca^{2+} through glutamate receptors can lead to an increased Ca^{2+} load in the mitochondria [69--71]. This uptake is dependent on the mitochondria possessing a membrane potential [69, 71]. Up to a certain point, this accumulation may be beneficial to the cell, since it decreases the cytoplasmic concentration of Ca^{2+}. However, excessive Ca^{2+} accumulation within this organelle can become deleterious, since it induces an abrupt dissipation in the mitochondrial transmembrane potential via MPT and respiratory impairment [71--73]. It is also possible that MPT induced by excessive matrix Ca^{2+} plays a role in neuronal cell

death [74, 75] perhaps causing release of apoptogenic proteins such as cytochrome c from the intermembrane space [76]. In fact, there have been a number of reports concerning the neuroprotective effects of the MPT inhibitor cyclosporin A [77--80]. However, cyclosporin A is also known to inhibit calcineurin [81] and has been reported to be neurotoxic [82], so these finding must be considered with caution. In at least one case, neuroprotection was not seen with FK506, which inhibits calcineurin but not the permeability transition [79].

Mitochondria Play a Role in Apoptosis of Mature Neurons

Mature neurons do not normally die unless they receive some toxic insult or they are diseased or aged. However, they can be induced to die by a wide variety of stimuli such as excitatory amino acids [57, 83--85], microtubule disruption [86, 87], growth factor deprivation (a model for developmental PCD) [88] and toxins such as 1-methyl-4-phenylpyridinium (MPTP) [89]. Neuronal death is associated with an increase in caspase activity in many of these cases including exposure to glutamate [85], colchicine [87] and MPTP [89], thus implicating apoptosis as the mode of neuronal cell death.

It has been shown that a number of these inducers of cell death are associated with the loss of cytochrome c from the mitochondria as part of the death program. Sympathetic neurons or superior cervical ganglion neurons exhibit a redistribution of cytochrome c from the mitochondria to the cytoplasm [90--92]. Similarly, neurotoxicity in rat cerebellar granule cells due to treatment with glutamate, colchicine, MPTP or potassium deprivation involves cytochrome c release from mitochondria [87, 89, 93, 94]. In the absence of evidence to the contrary, these findings clearly implicate participation of the apoptosome complex in neuronal cell death.

While there is an ongoing controversy concerning the role of apoptosis in certain neurological disorders (although evidence is mounting to support such a role), there is little doubt that mitochondrial dysfunction contributes to certain neurological disorders, including Parkinson’s disease [95], Alzheimer’s disease [96], Huntington’s disease [97] and ischemia [98].

Neuronal death in ischemia is due to the temporary deprivation of oxygen and glucose which leads to a cascade of events, including glutamate release and

excitotoxicity, resulting in cell death via both apoptosis and necrosis [99]. As previously mentioned, excitotoxicity is largely due to the massive increase in calcium ions that occurs, and it is becoming increasingly evident that the mitochondria play an important role in buffering this rise in Ca^{2+} [70, 71]. Apart from excitotoxicity, another component of ischemic death is the direct effect of reduced energy levels due to the diminution of oxygen and glucose supply. We and others have shown that hypoxia can directly induce apoptosis in neuronal [99--101] and nonneuronal cells [102]. In our studies of the mechanisms of neuronal cell death in hypoxia, in order to have a pure population of neurons, we chose to use a cell line, SK-N-MC, rather than primary neuronal cultures, where it is not possible to obtain a pure culture; the exception is cerebellar granule cell preparations, where the level of contamination with other cell types can be as low as 5--10%. However, it is well known that exposure of the cerebellar granule neurons to hypoxic conditions can lead to the release of glutamate and subsequent excitotoxicity [57, 83--85, 99]. Therefore, death due to hypoxia (apart from excitotoxicity) is more easily studied in a cell line which is not susceptible to excitotoxic death. Hypoxic cell death involves group 2 caspase activation, as judged by an increase in DEVD-MCA cleavage activity and cleavage of the caspase substrate PARP (fig. 2a, b) [100, 102]. This is also confirmed by depletion of procaspase 3, with a concomitant increase in the active form of the enzyme p17 (fig. 2c) [100] and the release of mitochondrial cytochrome c into the cytosol [100]. The intracellular trigger for hypoxia-induced death is unknown. Lowered oxygen levels are known to cause depression of mitochondrial protein synthesis; however, this effect is too rapid (within minutes to hours) to account for the induction of apoptosis over a time scale of days. It seems likely that death due to hypoxia may not be a direct result in the inhibition of mitochondrial respiration or of protein synthesis, but rather a consequence of the depletion of glycolytic substrates in the medium. This view is supported by the fact that compounds such as pyruvate/malate, which delay the depletion of ATP during oxygen deprivation, are protective against hypoxic neuronal damage [103]. It is not clear how these findings fit in with others that ATP/dATP is necessary for apoptosis [17, 55], although the levels of ATP in hypoxic cells may be low enough to trigger death yet sufficiently high to support apoptosis. Overall, however, these data show that neuronal cell death due to ischemia can occur via excitotoxicity involving Ca^{2+} overload of the mitochondria and/or as a result of direct energy impairment.

Parkinson's disease can be mimicked by administration of the neurotoxin MPTP [104]. The active form of MPTP is MPP^+ which causes a decline in mitochondrial respiration at the level of complex I, resulting in a rapid fall in ATP level and eventual cell death due to energy failure [105]. In the substantia nigra of Parkinson patients, complex I activity is found to be impaired (in the absence of changes in any other mitochondrial complexes) [95]. The reason for this decline is unknown, since no structural abnormalities have been found in complex I proteins, and no mitochondrial DNA mutations have so far been uncovered that would explain an inhibition of enzyme activity in Parkinson's disease [106]. A reduction in complex I activity could contribute to a cascade of events, including a reduction in ATP supply for various cellular processes, reduced efficiency of ion-motive ATPases and increased oxidative stress.

Although research into Alzheimer's disease has been dominated by studies on mutations in amyloid precursor protein and presenilins, there is also a link between late-onset Alzheimer's disease and heritable mutations in mitochondrial DNA encoding cytochrome c oxidases I and II, components of the electron chain complex [107]. This could explain the observations of increased levels of oxidative stress [108] and reduced energy availability to cells [109] in patients with Alzheimer's disease. Moreover, since agents that cause oxidative stress increase the production of β-amyloid [110] and promote its aggregation [111], mitochondrial DNA mutations could increase the risk of developing Alzheimer's disease.

Apoptosis during Brain Development

During embryonic development and postnatally, the neuronal cell number in the brain is finely tuned through the selective culling of superfluous neurons. This particular kind of death (apoptotic in nature) is more precisely known as programmed cell death, which refers to the temporally and spatially reproducible loss of cells during the development of an organism [112, 113]. Depending on the neuronal subpopulation, approximately 20--80% of all neurons produced during embryogenesis die before reaching adulthood [114]. Survival of neurons during the developmental period is dependent on trophic factors secreted either by the target cells that these neurons innervate or by other cells. Competition for these trophic factors results in survival of only a proportion of the neuronal population, thus

matching the size of the target cell population with the number of innervating neurons [115].

Studies with knockout animals provide evidence that the cytochrome c pathway is important in developmental neuronal apoptosis. However, this does not exclude other mechanisms. Elimination of functional Apaf-1 in mice has been demonstrated to cause brain malformations which result from excessive numbers of cells [116, 117]. Since cytochrome c has thus far been shown to be the only molecule capable of inducing dimerization of Apaf-1, leading to assembly of the apoptosome, this suggests that the cytochrome c pathway is involved in neuronal apoptosis in development. Similar phenotypes have been observed in caspase-9-deficient animals [9, 10]. This caspase has been shown to be the apical caspase in the proteolytic cascade initiated by cytochrome c [17, 118], again implicating this pathway in brain development. However, this does not exclude the possibility that other factors may replace cytochrome c in caspase 9 activation. Kuida et al. [9] have described the phenotype of caspase 9 null mice as follows: ‘there is severe malformation of the brain. A defect of neural tube closure in the hindbrain region was apparent at E10.5. At E13.5 the hindbrain neural tube remained open, and there was expansion of the midbrain region underneath the fetal skin tissue. By E16.5 there was prominent expansion (exencephaly) and protrusion of the entire cranial tissues.’ At the histological level, embryos exhibited prominent and ubiquitous enlargement of the proliferative zone in both the forebrain and midbrain. At E13.5, an expanded proliferative population was evident in the telencephalon, with both the lateral and third ventricles obstructed in the mutants [9]. Further evidence supporting the importance of this pathway to brain development comes from caspase 3 null mice, where there is decreased neuronal apoptosis such that the overall brain mass is substantially larger than that of wild-type mice. A variety of hyperplasias in the cerebellum, cortex, hippocampus and striatum are also present, and there is an absence of apoptotic cells at sites of major morphogenetic change during normal brain development [9]. However, in the case of caspase 3, it should be noted that there are other mechanisms by which it can be activated apart from the cytochrome c pathway (e.g. the Fas-mediated pathway).

Concluding Remarks

It is clear that mitochondria, once believed to function solely to the advantage of a cell, also play a pivotal role in cell death at a number of different levels. They have been suggested as the integrators of cell death pathways. Neuronal cells are not exempt from the negative properties of these Janus-like organelles, and in fact, because of their mobility, neuronal mitochondria are ideal sensors of death signals that impinge on widely spaced regions such as the cell body, neurites and synapses. Mitochondria can affect neuronal cell survival in a number of ways. The release of apoptogenic proteins to form the apoptosome most likely occurs during developmental neuronal cell death, and also death due to neurotoxic agents and hypoxia/ischemia. The findings by Shimizu et al. [26] that Bcl-2 family members interact with VDAC to modulate the release of cytochrome c promote VDAC as a potential therapeutic target for preventing apoptosis. Ca^{2+} buffering by the mitochondria is known to play an important role in excitotoxicity, while altered energy metabolism, possibly due to mutations in mitochondrial proteins, may contribute towards various neurodegenerative disorders.

Experimental Procedures

Materials

Unless otherwise indicated, all chemicals were purchased from Sigma. Dulbecco's modified Eagle Medium (DMEM) was from Gibco BRL. Ac-Asp-Glu-Val-Asp-α-(4-methyl-coumaryl-7-amide; DEVD-MCA) was from Peptide Institute Inc. (Mino, Japan). Anti-PARP monoclonal antibody was from Biomol (Plymouth Meeting, Pa., USA). Polyclonal rabbit antibody against the p17 fragment of caspase 3 was a gift from Dr. D.W. Nicholson (Merck Frosst Center for Therapeutic Research, Montreal, Canada).

Cell Culture

Human SK-N-MC neuroepithelioma cells were maintained in DMEM containing 10% fetal calf serum at 37 °C in a humidified 5% CO_2 atmosphere. They were routinely passaged once weekly. Unless otherwise indicated, cells for experiments were seeded at a density of 10^5 cells per cm^2 and cultured for a period of 1 day prior to exposure to hypoxia.

Exposure to Hypoxia

Cells were placed into a hypoxia chamber (Eurometric Instruments, Stockholm, Sweden) which was filled with a mixture of N_2/CO_2 (95/5%). The level of oxygen was maintained at 0--0.3% for the duration of the exposure. At the indicated times, the cells were removed and processed either for caspase assay or Western blotting.

Assessment of DEVD-MCA (i.e. Caspase 3 Like) Cleavage Activity

DEVD-MCA cleavage activity was measured as described by Nicholson et al. [3]. Briefly, cells were removed from the plastic dishes by gentle scraping, washed once with PBS and resuspended in PBS. Samples (25 µl) were incubated with 50 µl of assay buffer (100 m*M* HEPES, pH 7.25, 10% sucrose, 0.1% CHAPS, 5 mM dithiothreitol (DTT), 10^{-4}% Nonidet P-40) containing 50 µ*M* DEVD-MCA. Substrate cleavage leading to the release of free MCA (excitation 355 nm, emission 460 nm) was monitored at 37 ^{o}C using a Fluoroskan II (Labsystems AB, Stockholm, Sweden). Fluorescent units were converted to picomoles of MCA released using a standard curve generated with free MCA and subsequently related to protein content which was determined using the BioRad kit with bovine serum albumin as standard.

Western Blot Analysis

The cells were solubilized in buffer containing 20 m*M* Hepes, pH 7.5, 350 m*M* NaCl, 1 m*M* $MgCl_2$, 0.5 m*M* EDTA, 0.1 m*M* EGTA, 1% Nonidet P-40, 0.5 m*M* DTT, 0.2 m*M* phenylmethylsulphonyl fluoride and 2 µg/ml aprotinin. Protein content was determined using the Pierce kit with bovine serum albumin as standard. Samples were then solubilized in Laemmli's SDS-PAGE sample buffer and boiled for 5 min. Proteins (20--30 µg protein per lane) were then resolved on SDS-PAGE gels and electrophoretically transferred to nitrocellulose for 2 h at 100 V. Membranes were blocked overnight in 50 m*M* Tris, pH 7.5, with 500 m*M* NaCl, 1% bovine serum albumin and 5% nonfat dried milk. They were then probed for 1 h with one of the following antibodies: anti-p17 rabbit polyclonal antibody (1:1,000); anti-PARP mouse monoclonal (1:5,000). This was followed by 1 h incubation with appropriate secondary IgG conjugated to horseradish peroxidase (1:10,000) and then visualized using the Enhanced Chemiluminescence (ECL) Western Blot Detection kit from Amersham Corp. (Little Chalfont, UK).

Acknowledgments

We would like to acknowledge the kind gift of anti-p17 from Dr. D.W. Nicholson. This work was supported by a grant from the Swedish Fund for Research without Animals (CFN) and by a fellowship (to A.G.) from the Karolinska Institute.

Fig. 1. Schematic diagram showing release of cytochrome c from the mitochondria and activation of the caspase cascade. Apoptotic stimuli are transduced into a signal that induces translocation of apoptogenic proteins, including cytochrome c, from mitochondria. The release is either through a pore formed by VDAC whose opening is enhanced by the proapoptotic protein Bax and inhibited by Bcl-2 and Bcl-x_L (left hand side of scheme), or as a result of swelling of the matrix (with or without MPT) and rupture of the outer mitochondrial membrane (right hand side of the scheme). Cytochrome c, in combination with Apaf-1 then causes processing of procaspase 9 to the mature active form.

Fig. 2. Induction of caspase activity by hypoxia. **a** SK-N-MC cells were exposed to hypoxia for the indicated periods and DEVD-MCA cleavage activity was measured. Values are the mean $\pm$ SE of four separate determinations. **b** SK-N-MC cells were exposed to hypoxia. Whole cell lysates were made at the indicated times, and 25 µg of proteins was separated on a 12% SDS-PAGE. Cleavage of PARP was detected by Western blot analysis. **c** The blot was reprobed, and procaspase 3 levels were analyzed using anti-p17 antibodies.

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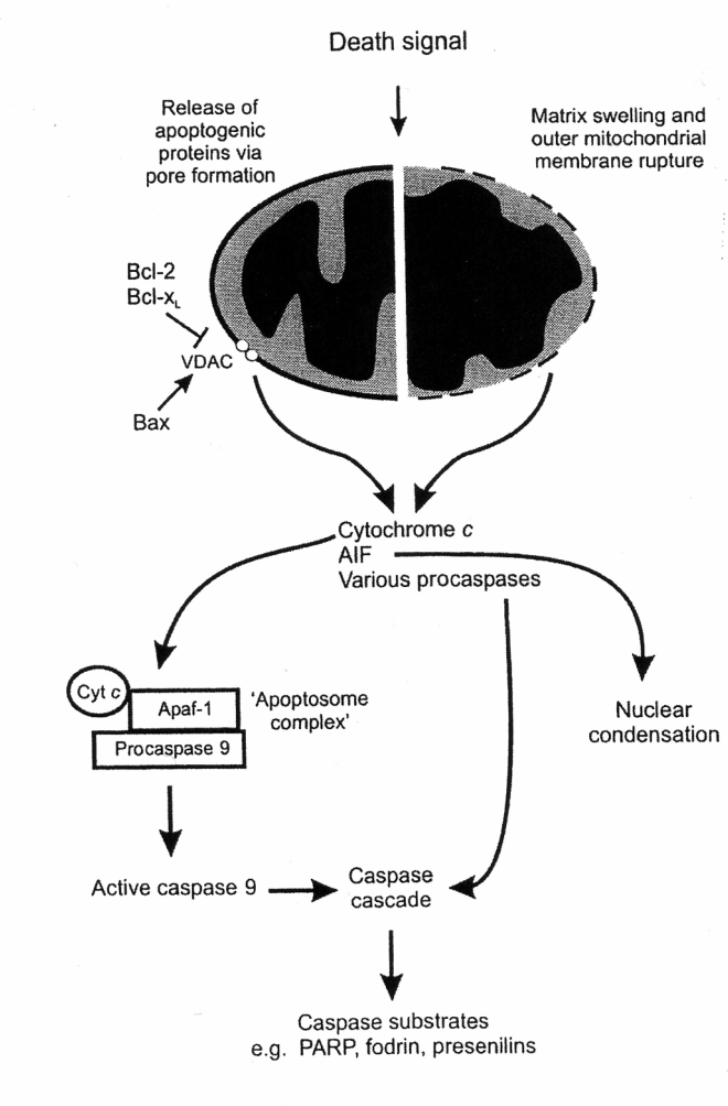
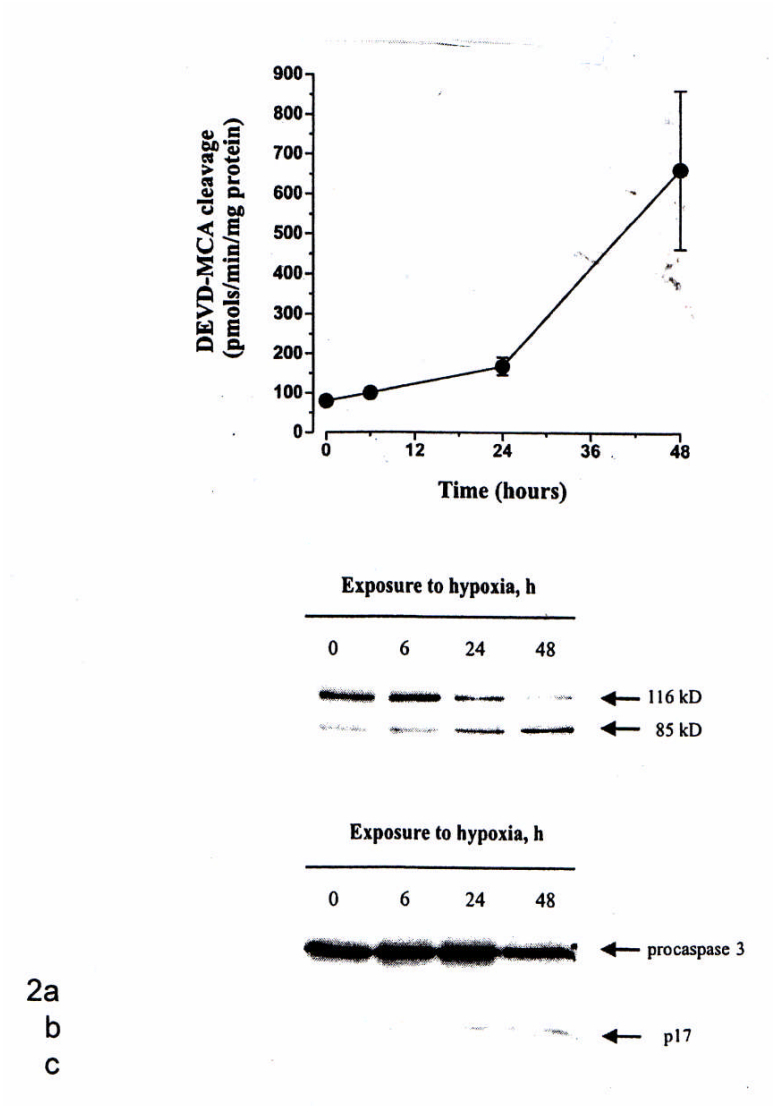

Death signal
Release of apoptogenic proteins via pore formation
Matrix swelling and outer mitochondrial membrane rupture
Bcl-2
Bcl-x_L
VDAC
Bax
Cytochrome *c*
AIF
Various procaspases
Cyt *c*
Apaf-1
Procaspase 9
'Apoptosome complex'
Nuclear condensation
Active caspase 9
Caspase cascade
Caspase substrates
e.g. PARP, fodrin, presenilins


DEVD-MCA cleavage
(pmols/min/mg protein)
900
800
700
600
500
400
300
200
100
0
0
12
24
36
48
Time (hours)
Exposure to hypoxia, h
0
6
24
48
116 kD
85 kD
Exposure to hypoxia, h
0
6
24
48
procaspase 3
p17

2a

b

c