Neurochemical Research, Vol. 28, No. 10, October 2003 (© 2003), pp. 1563–1574

Mitochondrial Dysfunction and Reactive Oxygen Species in Excitotoxicity and Apoptosis: Implications for the Pathogenesis of Neurodegenerative Diseases*

A. Cristina Rego[1,2] and Catarina R. Oliveira[1]

(Accepted April 2, 2003)

In recent years we have witnessed a major interest in the study of the role of mitochondria, not only as ATP producers through oxidative phosphorylation but also as regulators of intracellular Ca^{2+} homeostasis and endogenous producers of reactive oxygen species (ROS). Interestingly, the mitochondria have been also implicated as central executioners of cell death. Increased mitochondrial Ca^{2+} overload as a result of excitotoxicity has been associated with the generation of superoxide and may induce the release of proapoptotic mitochondrial proteins, proceeding through DNA fragmentation/condensation and culminating in cell demise by apoptosis and/or necrosis. In addition, these processes have been implicated in the pathogenesis of many neurodegenerative diseases, which share several features of cell death: selective brain areas undergo neurodegeneration, involving mitochondrial dysfunction (mitochondrial complexes are affected), loss of intracellular Ca^{2+} homeostasis, excitotoxicity, and the extracellular or intracellular accumulation of insoluble protein aggregates in the brain.

KEY WORDS: Apoptosis; calcium homeostasis; excitotoxicity; mitochondrial depolarization; neurodegenerative diseases; oxidative stress.

INTRODUCTION

Mitochondrial dysfunction associated with the loss of Ca^{2+} homeostasis and enhanced cellular oxidative stress have long been recognized to play a major role in cell damage associated with excitotoxicity (1), a process resulting from the overstimulation of glutamate receptors. Under this perspective, the stimulation of ionotropic glutamate receptors responsible for a major Ca^{2+} entry, in particular, the *N*-methyl-D-aspartate (NMDA) receptors

* This manuscript is dedicated to Professor Arsélio Pato de Carvalho. As a scientist he represents the highest standards of scientific integrity and research. As the head of the Center for Neuroscience and Cell Biology at the University of Coimbra he has always encouraged younger scientists to pursue their ideas. His great enthusiasm for research in Neuroscience has been a great motivation for all of us.

[1] Institute of Biochemistry, Faculty of Medicine and Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal.

[2] Address reprint requests to: Institute of Biochemistry, Faculty of Medicine and Center for Neuroscience and Cell Biology, University of Coimbra, 3004–504 Coimbra, Portugal. Tel: 351–239–820190; Fax: 351–239–822776; E-mail: acrego@cnc.cj.uc.pt

ABBREVIATIONS: AD, Alzheimer's disease; AIF, apoptosis-inducing factor; AIGP1, axotomy-induced glycosylated/Golgi complex protein 1; ALS, amyotrophic lateral sclerosis; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; Apaf-1, apoptotic protease activating factor-1; APP, amyloid precursor protein; Bad, Bcl-2-associated death protein; Bax, Bcl-2–associated X protein; Bcl-2, B-cell leukemia/lymphoma 2; Diablo, direct IAP binding protein with low pI; DFF, DNA fragmentation factor; ER, endoplasmic reticulum; FADD, Fas-associated protein with death domain; H_2O_2, hydrogen peroxide; HD, Huntington's disease; $HO^{\cdot}$, hydroxyl radical; IAP, inhibitor of apoptosis protein; ICAD, inhibitor of caspase-activated DNase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NMDA, *N*-methyl-D-aspartate; $O_2^{\cdot-}$, superoxide anion; PARP, poly(ADP-ribose)polymerase; PD, Parkinson's disease; PI3K, phosphatidylinositol-3-kinase, PS, presenilin; PSAP, presenilin-associated protein; PTP, permeability transition pore; ROS, reactive oxygen species; Smac, second mitochondrial activator of caspases; SOD, superoxide dismutase; STS, staurosporine; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; VDAC, voltage-dependent anion channel.

0364-3190/03/1000–1563/0

and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors lacking the GluR2 subunit, are highly implicated in the process of neuronal death (e.g., [2–5]). Energy depletion and increased oxidative damage to several synaptic proteins may result in loss of local Ca^{2+} homeostasis, and, in consequence, synaptic degeneration occurs. Calcium is known to activate several intracellular enzymes, such as phospholipase A_2, nitric oxide synthase, xanthine dehydrogenase, calcineurin, and endonucleases, many of which can elicit the generation of endogenous ROS. Moreover, when taken up by the mitochondria, Ca^{2+} can physiologically increase ATP generation by activating matrix dehydrogenases (6). However, an increase in mitochondrial Ca^{2+} can also promote ROS generation (7).

Neurons are highly dependent on glucose for ATP generation necessary for many biochemical processes and produce ROS as by-products of the oxidative phosphorylation within the mitochondria. Inevitably, if the amount of ROS produced unbalances the few antioxidants, oxidative stress occurs, followed by neuronal damage. The CNS is particularly susceptible to ROS-induced damage (reviewed in [8]) because (i) it has a high consumption of oxygen; (ii) it contains high levels of membrane polyunsaturated fatty acids susceptible to free radical attack; (iii) it is relatively deficient in oxidative defenses (poor catalase activity and moderate superoxide dismutase, SOD, and glutathione peroxidase activities); and (iv) a high content in iron and ascorbate can be found in some regions of the CNS, enabling the generation of more ROS through the Fenton/Haber Weiss reaction. Moreover, ROS have been demonstrated to mediate neuronal death in several neurodegenerative diseases such as Parkinson's (PD), Alzheimer's (AD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD).

Intracellular Ca^{2+} overload associated with excitotoxicity has long been described to induce both apoptosis and necrosis (9), although the exact distinction between these two processes is not always clear. Apoptosis is a highly regulated cellular suicide program with distinct morphological features, required for embryonic development, for maintenance of cellular homeostasis, and occurring in many degenerative diseases. The biochemical machinery involved in apoptosis is conserved from *Caenorhabditis elegans* and *Drosophila* to mammals. In addition, many proapoptotic stimuli converge on mitochondria, inducing the increase in permeabilization of the outer mitochondrial membrane. Thus a key event in apoptosis is the release of cytochrome c to the cytosol, although the release of other mitochondrial factors also seems to help regulate the apoptotic machinery through a process dependent on or independent (e.g., the apoptosis-inducing factor or AIF) of caspases activation. These processes culminate with DNA fragmentation and condensation. The slow, cascade-dependent apoptotic cell death process has been implicated to occur in several neurodegenerative diseases (10), although its involvement is still not clear in all these disorders, of which one of the most controversial is HD.

Here we summarize some of the most relevant hallmarks of the cell death process, with the main emphasis on apoptosis, along with recent advances in the mechanisms involved. The analysis of the role of mitochondria in the regulation of Ca^{2+} homeostasis and in ROS generation during excitotoxicity and general cell demise also will be discussed. Finally, the relevance and involvement of some of the basic features of neuronal demise in three neurodegenerative diseases, PD, AD, and HD, will be addressed.

DISCUSSION

Involvement of the Mitochondria in the Apoptotic Machinery

Death Receptor or Mitochondrial Apoptotic Pathways. Two main apoptotic pathways have been identified: the pathway involving the activation of death receptors and the mitochondrial pathway, both involving the activation of a caspase cascade (Fig. 1).

The death signals tumor necrosis factor (TNF) or Fas ligand interact with the death receptors at the plasma membrane, resulting in the recruitment of adaptor molecules such as the Fas-associated protein with death domain (FADD), which are responsible for activating caspase-8 through the interaction of the death effector domains. Activated caspase-8 can directly activate caspases-3 and -7 (effector caspases), but it can also cleave Bid, a BH3-only member of the B-cell leukemia/lymphoma 2 (Bcl-2) family of proteins. The cleaved C-terminal Bid (truncated Bid or tBid) translocates to the mitochondria and induces the release of (holo)cytochrome c (Fig. 1), linking the death receptor pathway with the mitochondrial pathway (11). Interaction of tBid with the mitochondria does not seem to require the activation of the permeability transition pore (PTP) or the proapoptotic protein Bcl-2–associated X protein (Bax), although tBid and Bax can function synergistically (12). In addition, Bid-induced cytochrome c release can be antagonized by Bcl-2, a cell death repressor protein (11).

Many death stimuli (e.g., growth factor deprivation, ionizing radiation, and several chemical agents) activate the mitochondrial pathway by inducing the release of

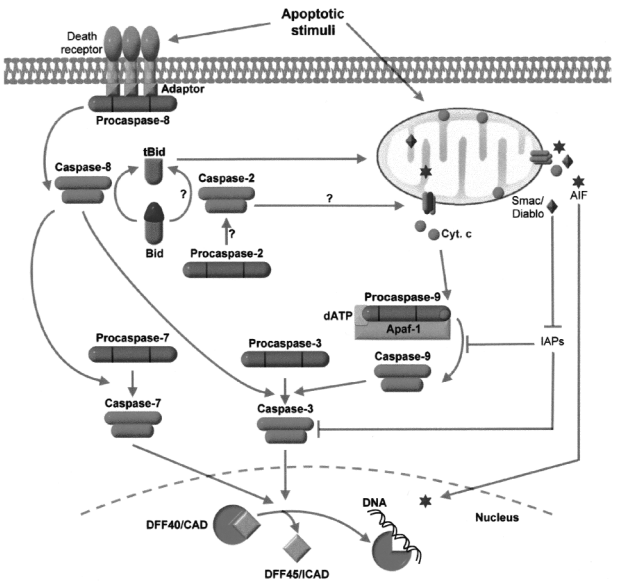

Fig. 1. Schematic overview of the death receptor and mitochondrial apoptotic pathways. External ligands interact with death receptors, activating the initiator caspase-8, which can cleave Bid. tBid translocates to the mitochondria and induces the release of cytochrome c (cyt c) to the cytosol, linking the death receptor pathway with the mitochondrial pathway. Activation of caspase-2 may also induce Bid cleavage and signal the mitochondria to release cyt c, implicating the mitochondria as amplifiers of the caspase cascade. Within the cytosol, cyt c forms the apoptosome (in the presence Apaf-1, dATP and procaspase-9) activating caspase-9. The effector caspases -3 and -7, which can be activated by caspases -8 or -9, are required for the activity of the endonuclease DFF40/CAD, responsible for genomic DNA fragmentation. In addition, Smac/Diablo (released from the mitochondria) binds to IAPs, potentiating caspase activation. AIF is also released to the cytosol and translocates to the nucleus, inducing the fragmentation of DNA and killing the cells in a caspase-independent manner.

(holo)cytochrome c. Within the cytosol, cytochrome c initiates the formation of the apoptosome, a multimeric complex, after binding to the apoptotic protease activating factor-1 (Apaf-1) in the presence of deoxyATP. This complex is responsible for activating caspase-9 (Fig. 1). Curiously, the apocytochrome c (lacking the heme) still binds Apaf-1, but blocks caspase-9 activation and Bax-induced apoptosis (13). Then the initiator caspase-9 activates the downstream caspases -3, -6, and -7. Caspase-3 (and also caspase-7) is required for the cleavage of the 45-kD subunit of the DNA fragmentation factor (DFF) or DFF45 (ICAD or inhibitor of caspase-activated DNase is the mouse homologue), releasing the DFF40 (CAD is the mouse homologue) with nuclease activity, responsible for DNA fragmentation during apoptosis (14) (Fig. 1).

Apart from cytochrome c, the increase in permeability of the outer mitochondrial membrane may also mediate the release of the apoptosis-inducing factor (AIF) (15), a phylogenetically old flavoprotein that can stably bind FAD ([16], for review). After an apoptotic insult, this protein is released to the cytosol and translocates to the nucleus, where it binds to the DNA and induces its fragmentation, independently of the activity of caspases (Fig. 1). A similar function was associated to endonuclease G, a mitochondria-specific nuclease (present in the intermembrane space) that translocates to the nucleus and cleaves chromatin DNA during apoptosis, killing the cells in a caspase-independent manner (17).

Similar to cytochrome c, the second mitochondrial activator of caspases (Smac)/direct IAP binding protein with low pI (Diablo), a protein that binds to inhibitor of apoptosis proteins (IAPs), is released from the mitochondria and potentiates apoptosis by relieving IAP inhibition of caspases (Fig. 1), as identified by Verhagen et al. (18) and Du et al. (19). Smac/Diablo, like the serine protease Omi/Htr2A (also released from the mitochondria), interacts with several IAPs and mediates the activation of the initiator caspase-9 and the effector caspases -3, -6, and -7. Interestingly, release of Smac can occur before or in parallel with cytochrome c, in a process modulated by the antiapoptotic proteins Bcl-2 and Bcl-XL upon apoptosis induced by the TNF-related apoptosis-inducing ligand (TRAIL) (20). In cells exposed to staurosporine (STS), the time required for Smac/Diablo release was shown to be about four times longer than that required for cytochrome c, suggesting that distinct pathways for exit from the mitochondria could be used by these proteins (21). STS is a protein kinase C inhibitor that induces apoptosis in a variety of cells (22–25), although the exact mechanism is still not clear.

The ubiquitous caspase-2 was one of the first caspases to be identified; however, the mechanisms involved in caspase-2 activation and related Bcl-2 protection are not completely understood yet. Recent important contributions to this field were made by Lassus et al. (26). They found that caspase-2 is required for translocation of Bax to the mitochondria and release of cytochrome c and Smac/Diablo. Other authors have also reported that caspase-2 induces the release of AIF and that Bid may also be involved (reviewed in [27]). These, together with data referring that caspase-2 in the nucleus signaled the mitochondria to release cytochrome c, implicated the mitochondria as amplifiers rather than initiators of caspase activity (26,27).

An interesting observation is that increased mitochondrial inner membrane permeability, defined by the formation of the PTP, has been suggested to be involved not only in apoptotic but also in necrotic cell death, and in autophagy, a less studied form of cell death that may

be implicated in removing damaged mitochondria (28). Thus the occurrence of necrosis or apoptosis-type of cell death may depend upon the intracellular levels of ATP, as accepted some years ago: if the ATP levels fall, plasma membrane rupture occurs; if ATP levels are maintained, the caspase-dependent apoptotic machinery can proceed. In serum deprived PC12 cells, with recognized apoptotic features, Uchiyama (29) has described the occurrence of autophagy, regulated by lysosomal proteinases, cathepsins B and D, in the early stages of apoptosis, before the appearance of nuclear changes. Moreover, apoptosis was prevented in the presence of an autophagy inhibitor (29).

Bcl-2 Related Proteins: Bax, Bad, and Bcl-2. The Bcl-2 family members are key players in the apoptotic process. Some members of this family promote cell survival (Bcl-2 or Bcl-XL), whereas others are proapoptotic (Bax, Bak, Bad, Bik, and Bim).

The proapoptotic Bax was previously reported to mediate the release of cytochrome c through the opening of the PTP, in a process regulated by cyclosporin A (30). However, Bax was also demonstrated to trigger cytochrome c release from isolated mitochondria independently of the Ca^{2+}-inducible, cyclosporin A-dependent PTP (31). More recently, Bax was shown to be translocated from the cytosol to the mitochondria forming homo-oligomers responsible for cytochrome c release (32).

Activation of the receptors for neurotrophins plays an important role in regulating the apoptotic activity of the BH3-only proteins. One known example is the regulation of the Bcl-2–associated death protein (Bad). Phosphorylation of Bad at Ser^{112} and Ser^{136}, namely by the phosphatidylinositol-3-kinase (PI3K)/Akt (protein kinase B) signaling pathway, mediates its sequestering by the cytosolic protein 14-3-3, avoiding its heterodimerization with Bcl-2 and Bcl-XL at the mitochondrial membrane (Fig. 2). In contrast, in the presence of an apoptotic stimuli (e.g., Ca^{2+}-calmodulin induced activation of calcineurin or protein phosphatase 2B), dephosphorylated Bad is released from the 14-3-3 anchor and is translocated to the mitochondria (Fig. 2), free to execute its proapoptotic activity. By binding to Bcl-2 or Bcl-XL, the antiapoptotic function of these proteins is no longer exerted, allowing the release of cytochrome c to the cytosol. Seizure-induced hippocampal neuronal death was recently associated with the activation of Bad cell death pathway (33). Moreover, in traumatic spinal cord injury, the apoptotic cascade involving caspase-3 activation was shown to depend upon calcineurin-mediated Bad dephosphorylation (34). In recent studies, calcineurin was also implicated in 3-nitropropionic acid–induced cytochrome c release and caspase-3 activation in cultured cortical and striatal neurons (Almeida, Brito, Oliveira, and Rego, unpublished results).

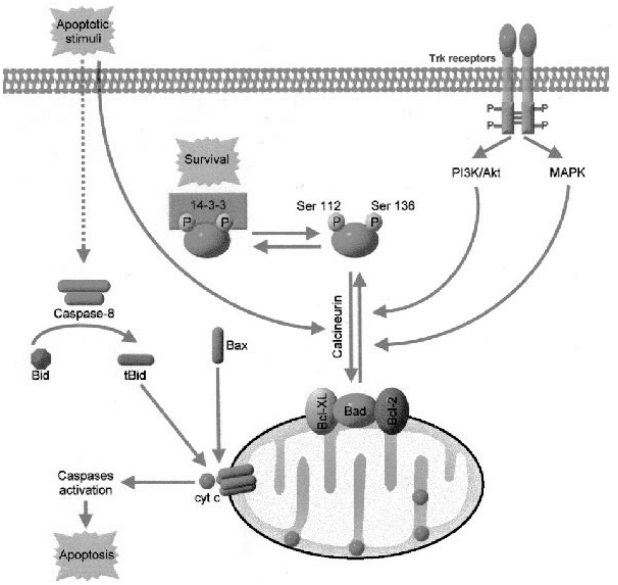

Fig. 2. Regulation of apoptosis by Bad phosphorylation/dephosphorylation. In the presence of an apoptotic stimuli (e.g., Ca^{2+}-calmodulin), calcineurin dephosphorylates Bad, which is translocated to the mitochondria, where it interacts with Bcl-2 or Bcl-XL. Under these conditions, release of cytochrome c (cyt c) to the cytosol occurs, triggering the apoptotic machinery through the activation of the caspase cascade. Activation of the receptors for neurotrophins (Trk receptors) activates the PI3K/Akt or MAPK signaling pathways, responsible for phosphorylating Bad. Phosphorylated Bad at Ser^{112} and Ser^{136} is sequestrated by the cytosolic protein 14-3-3, avoiding its translocation to the mitochondria.

Bcl-2, known as ced-9 in *C. elegans*, is one of the antiapoptotic proteins first recognized to prevent the release of mitochondrial cytochrome c (35). Bcl-2 family members seem to be associated with the outer mitochondrial membrane, the endoplasmic reticulum (ER), and the nuclear envelope. Interestingly, expression of Bcl-2 targeted to the mitochondria was reported to induce apoptosis, whereas expression of this protein targeted to the ER was shown to be protective against Bax-induced cell death (36). Bcl-2 protection of Bax-induced apoptosis seems to be related with Bax remaining monomeric, although it may not depend on a physical interaction between the two proteins (32). In addition, Bcl-2 has been shown to inhibit intracellular formation of free radicals (37), shifting the redox potential of cells toward reduction (38), and increase the capacity of mitochondria to accumulate Ca^{2+} (39). Bcl-2 can be modulated by dimerization, for example with Bax, and by phosphorylation (by protein kinase C), although the role of Bcl-2 phosphorylation as a regulator of its antiapoptotic activity is still controversial.

Loss of Mitochondrial Potential and Apoptotic Cell Death. Opening of the PTP (to release cytochrome c) is activated by Ca^{2+}, ROS, and a decrease in mitochondrial membrane potential ($\Delta\Psi_m$). Nevertheless, mitochondrial depolarization is not always associated with the release of cytochrome c.

In PC6 cells, STS-induced apoptosis was associated with a concomitant release of cytochrome c (as a green-fluorescent–protein construct) and collapse of $\Delta\Psi_m$ (23). However, other authors have found that the release of cytochrome c into the cytosol occurs independently of mitochondrial depolarization during STS-induced apoptosis (22,40). In GT1–7 cells, we have previously observed that some mitochondria could lose their cytochrome c during STS-induced apoptosis by a mechanism independent of a collapse in $\Delta\Psi_m$ (25). By using oligomycin, we demonstrated that the cells maintained the $\Delta\Psi_m$ following cytochrome c release by ATP hydrolysis. In addition, we showed that STS decreased state-3 respiration, implicating the inhibition of electron transport chain and a consequent compromise in respiratory function, which could be in the basis for ATP hydrolysis (25). Similar to Zhu et al. (39), we have shown that overexpression of Bcl-2 prevented a decrease in mitochondrial potential elicited by STS (25).

Bid and Bad, two BH3-only proteins, but not Bax or Bak, were also shown to induce cytochrome c release without changes in $\Delta\Psi_m$ or the opening of the PTP and without interacting with the voltage-dependent anion channel (VDAC) (41).

The Mitochondria and the Endoplasmic Reticulum Crosstalk. Not only do mitochondria have a relevant role in apoptotic cell death, but other organelles, such as the ER, seem to be quite sensitive to perturbations in ion homeostasis, oxidative stress, or metabolic perturbations in the cell. It has long been recognized that mitochondria are capable of sequestering Ca^{2+} released from inositol trisphosphate–stimulated neighboring ER (42). Interestingly, some of the Ca^{2+} taken up by the mitochondria seems to be recycled back to refill the ER, as determined by using the "cameleon" indicators targeted to the cytosol, ER lumen, and mitochondrial matrix (43). Recently, it was demonstrated that the VDAC in the outer mitochondria membrane may enhance the transfer of Ca^{2+} from the ER to the inner mitochondria membrane and increase the sensitivity to apoptotic cell death induced by ceramide (44), involved as a second messenger in the sphingomyelin cycle. The ER stress involves the alteration of protein folding processes or the accumulation of malformed proteins. Caspase-12, an ER resident caspase, is activated during ER stress. Recent data suggest that neuronal apoptosis, determined by caspase-12 activation and nuclei fragmentation, can be induced by ER stress in the presence of brefeldin A, an inhibitor of ER to Golgi protein transportation, through the activation of the NF-kB pathway (45). In damaged neurons, the increased expression of the resident Golgi protein axotomy-induced glycosylated/Golgi complex protein 1 (AIGP1) by ER stress inducers may contribute to interorganelle signaling during apoptotic neuronal death (46). ER stress–mediated cell death was also demonstrated to require translocation of caspase-7 to the ER surface, necessary for the cleavage of the prodomain of caspase-12; in addition, caspase-12 was reported to move into the cytoplasm upon a prolonged ER stress, interacting with caspase-9 (47), highly suggesting the coupling between an "ER-apoptotic pathway" with the known mitochondrial pathway.

In fact, an intricate interorganelle cross-talk was previously suggested by Ferri and Kroemer (48), who reviewed the participation of distinct organelles, namely the nuclei, lisosomes, ER, and Golgi, in the release of (apoptotic) signals that converged in the mitochondria, the central executioner.

Excitotoxicity-Induced Mitochondrial Dysfunction and Cell Death

NMDA Excitotoxicity. Exposure of neurons to glutamate was previously demonstrated to result in mitochondrial depolarization associated with increased Ca^{2+} uptake into the mitochondria (e.g., 9,49–51).

Activation of NMDA receptors was reported to induce faster mitochondrial Ca^{2+} uptake and in a more tightly coupled way, compared to kainate or KC1. This observation suggested a privileged access to mitochondria of Ca^{2+} entering through NMDA receptors, which could be accounted for by the possibility that mitochondria are in closer proximity to NMDA receptors than other routes of Ca^{2+} entry (49). In previous work, we reported that NMDA receptor–dependent transient mitochondrial Ca^{2+} loading could initiate oxidative damage (e.g., affecting plasma membrane Ca^{2+} extrusion pathways, namely the Ca^{2+}-ATPase) and/or inhibit mitochondrial respiration, two factors suggested to precipitate delayed Ca^{2+} deregulation, a failure of the cell to maintain a low cytoplasmic free calcium concentration (51). In cultured retinal neurons, we have also observed a decrease in oxygen consumption upon glutamate exposure, coincident with the inhibition (by about 20%) in the activity of mitochondrial complexes I, II/III, and IV (52). Curiously, during continuous activation of the NMDA receptors, mitochondria depolarization occurred concomitantly with the delayed Ca^{2+} deregulation (51), which seems to precede the

subsequent necrotic death of the cell (2). In addition, in cells stimulated with glutamate and glycine, the mitochondria continued to generate ATP (53). Once started, the NMDA receptor–induced delayed, secondary Ca^{2+} rise was shown to be irreversible in cultured cerebellar granule cells, as determined upon exposure to antagonists of NMDA or non-NMDA glutamate receptors, Ca^{2+} channel blockers, or even in the presence of inhibitors of the PTP (53). Nevertheless, in striatal neurons Alano et al. (54) have recently shown that exposure to *N*-methyl-valine-4-cyclosporin, a blocker of the PTP, prevented the secondary cytosolic Ca^{2+} rise induced by NMDA receptor activation.

The occurrence of apoptosis upon NMDA excitotoxicity has been controversial. During excitotoxicity, the release of mitochondrial cytochrome c associated with a delayed mitochondrial depolarization and production of ROS were documented (55,56). Previous reports have also showed that caspase-3, in particular, plays a major role in NMDA excitotoxicity (57). Moreover, AIF translocation was observed upon the stimulation of the NMDA receptors in a process requiring the activation of poly(ADP-ribose)polymerase (PARP) and the consequent depletion of NAD^+ (58), although the mechanisms involved are still not completely clear.

AMPA Excitotoxicity. Both delayed Ca^{2+} deregulation and mitochondrial depolarization were found after kainate stimulation in cerebellar granule cells (59). Kainate is known to activate nondesensitizing AMPA receptors and mediate a large increase in cytosolic Na^+, suggesting an important role in cell swelling (60). However, we found that, in contrast with NMDA receptor activation, the mitochondria were not highly loaded with Ca^{2+} upon kainate stimulation in cerebellar granule cells (59). Nevertheless, mitochondrial Ca^{2+} loading upon kainate exposure was observed, for example, in spinal motor neurons (4). Inhibition of ATP synthase with oligomycin was shown to protect the cells against kainate-induced delayed Ca^{2+} deregulation, inducing a slow mitochondrial depolarization (59). These results indicated that $\Delta\Psi_m$ could be maintained by ATP synthase reversal, presumably through the hydrolysis of glycolytic ATP, highly suggesting the failure of oxidative phosphorylation.

Both necrotic and apoptotic types of cell death have been attributed to the neurotoxic effects of AMPA/kainate receptor stimulation, as reported in cerebellar granule cells (3) or cortical neurons (61). Recently, we have demonstrated that a brief stimulation of AMPA receptors under nondesensitizing conditions induces neuronal death by necrosis and apoptosis in hippocampal cultures, as determined by the loss of cell viability, the release of cytochrome c, the activation of caspases -1 and -3, and DNA condensation/fragmentation, without significant changes in intracellular ATP/ADP levels (62). In addition, kainate excitotoxicity was shown to be related to the induction of Bax, the release of cytochrome c, and the activation of several caspases in hippocampal slice cultures, together with a p53-dependent neuronal death (5). Importantly, secondary activation of NMDA receptors, stimulated by depolarization-induced removal of Mg^{2+} blockade, can occur after AMPA-mediated cytosolic Na^+ loading (63), giving rise to a large percentage of neurons entering into necrosis.

The Deleterious Power of ROS in Excitotoxicity and Apoptosis

The superoxide anion ($O_2^{\cdot-}$) is generated in a constant manner by the mitochondria. The semiquinone form of coenzyme Q is an important electron leakage site in the inner mitochondrial membrane (64). Thus, increased Ca^{2+} uptake by the mitochondria was reported to stimulate oxygen radical production from the reduced form of coenzyme Q (7). In more recent studies, Maciel et al. (65) have determined that Ca^{2+}-induced opening of the PTP in brain mitochondria potentiates the formation of mitochondrial ROS and lipid peroxidation by depleting the levels of NAD(P)H.

Production of $O_2^{\cdot-}$ has been also associated with increased NMDA or kainate excitotoxicity, which can lead to the disruption of mitochondrial energy production and cell death, as evaluated by the inactivation of aconitase, a key enzyme in the TCA cycle (66). An enhancement in $O_2^{\cdot-}$ formation may also be in the basis for cytochrome c release, as reported by Atlante et al. (55) in cerebellar granule neurons undergoing glutamate-induced excitotoxicity. Otherwise, $O_2^{\cdot-}$ generation may occur secondary to cytochrome c release, as observed upon NMDA-neurotoxicity (56). In addition, activation of AMPA receptors was previously shown to enhance the production of mitochondrial $O_2^{\cdot-}$ (4,67). In recent studies, we have observed a moderate increase in $O_2^{\cdot-}$, without a detectable generation of intracellular peroxides after a brief desensitization of AMPA receptors in cultured rat hippocampal neurons (62). According to Luetjens et al. (56), delayed $O_2^{\cdot-}$ generation during excitotoxicity occurs secondarily to a defect in mitochondrial electron transport, downstream of mitochondrial cytochrome c release. Cai and Jones (68) have shown that $O_2^{\cdot-}$ is formed from mitochondria isolated from apoptotic cells upon the release of cytochrome c and

that Bcl-2 could protect from $O_2^{\cdot-}$ by preventing the release of cytochrome c, explaining the antioxidant properties (37) of this antiapoptotic protein.

The activity of SOD (Mn-SOD and/or Cu/Zn-SOD) controls the levels of $O_2^{\cdot-}$, producing hydrogen peroxide (H_2O_2). Similarly to $O_2^{\cdot-}$, H_2O_2 is not very reactive, unless it encounters Fe^{2+} or Cu^{+} to form the highly reactive and short-lived (about 10^{-9} s) hydroxyl radical ($HO^{\bullet}$) by the Fenton-Haber Weiss reaction. Importantly, an increased expression of Cu/Zn-SOD was shown to reduce oxidative stress and attenuate apoptotic cell death by decreasing mitochondrial cytochrome c and Smac/Diablo release and the activation of caspases (69), in agreement with previous studies showing that SOD delayed neuronal apoptosis induced by nerve growth factor deprivation (70). Sublethal exposure to H_2O_2 was shown to cause the formation of the PTP, resulting in mitochondrial membrane depolarization and cytochrome c release, which could be prevented by cyclosporin A, a blocker of the PTP (71).

During apoptosis induced by STS, increased intracellular Ca^{2+} concentration and enhanced formation of ROS have been observed (24,72), suggesting that the caspases could be redox regulated. Using retinal cell cultures, we determined that STS-mediated apoptotic death increased the production of intracellular peroxides and that distinct antioxidants, but not reducing agents, were able to prevent from caspases activation and DNA fragmentation (Gil, Oliveira and Rego unpublished results). Previously, the antioxidant trolox, a lipophilic analogue of vitamin E, was shown to protect against apoptosis induced by H_2O_2 by preventing DNA fragmentation (73). Vitamin E or the superoxide dismutase-mimetic MnT-BAP was shown to reduce apoptosis induced by STS in cultured rat hippocampal neurons (74). Moreover, STS-mediated glutathione depletion and apoptotic injury were shown to be prevented by vitamin E and retinoic acid in cultured chick embryonic neurons (75).

Features of Cell Death in Three Human Neurodegenerative Diseases: Parkinson's, Alzheimer's, and Huntington's Diseases

The pathogenesis of the majority of the neurodegenerative disorders involves a genetic predisposition together with the activity of environmental (mitochondrial) toxins. The accumulation of abnormally folded proteins, leading to the activation of heat-shock/stress responses and, particularly, the formation of insoluble protein aggregates (because the proteins adopt β-sheet structures) in the brain seem to be common features of several neurodegenerative diseases ([76], for review). Thus the amyloid-like fibrillary aggregates may arise in the intracellular or extracellular milieu in many neurodegenerative diseases. However, the nature of pathogenic aggregate formation and the exact consequences of their accumulation are still not clear. Moreover, there are still some controversies concerning the evidence for classical apoptosis in many neurodegenerative diseases, that is, AD, PD, ALS, and HD.

Parkinson's Disease. PD is characterized by a selective degeneration of the dopaminergic neurons of the substantia nigra and the formation of intracellular fibrillar α-synuclein and ubiquitin, two protein components of the Lewy bodies. Autosomal recessive juvenile forms of PD result from mutations in parkin, a ubiquitin protein ligase, shown to elevate oxidative stress (increased protein carbonyls and lipid peroxidation) and nitric oxide production upon expression, sensitizing to cell death processes (77). An apoptotic type of cell death involving the mitochondria has been suggested to occur in PD, concomitantly with the observation of a decrease in the activity of mitochondrial complex I, which may result in oxidative stress and increase the susceptibility of neurons to excitotoxic death. Alterations in mitochondrial function, activation of caspases, and cell death with apoptotic morphology have been described in several models of PD and in postmortem brains of PD patients. In addition, the degeneration of dopaminergic neurons cause a unique form of oxidative stress induced by the oxidation of dopamine, which can produce H_2O_2, via monoamine oxidase, or produce a quinone, upon oxidation of its catechol group.

Epidemiological studies have made clear that exposure to pesticides is associated with an increased risk of developing PD, suggesting a high relevance of environmental toxins in PD pathogenesis. The toxins rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) inhibit mitochondrial complex I, reproducing most of the features of PD by producing selective degeneration of dopaminergic neurons of the substantia nigra. Interestingly, chronic exposure to rotenone was reported to cause the accumulation and aggregation of α-synuclein and ubiquitin (78,79), progressive oxidative damage, and caspase-dependent cell death (79). Moreover, the α-synuclein inclusions disappeared after restoring the normal mitochondrial metabolism (78). Rotenone-induced apoptosis was also associated with an increase in the processing of procaspases -12, -9, and -3, suggesting that both mitochondria and ER-dependent caspases are activated (80).

α-Synuclein also appears to have an antiapoptotic role, as detected through a reduction in STS-induced

caspase-3 activation, DNA fragmentation, or p53 expression (81). However, the natural toxin 6-hydroxydopamine was shown to abolish this α-synuclein role, triggering its aggregation, a pathological hallmark of PD. Nevertheless, an earlier report indicated that overexpression of wild-type α-synuclein delayed cell death induced by H_2O_2 or serum withdrawal, attenuating the decrease in reduced glutathione, although aggravating the effects induced by STS (82). Expression of mutant α-synucleins, involved in some cases of familial PD, was shown to increase cellular oxidative stress, as determined through the oxidation of DNA, proteins, and lipids (82).

Alzheimer's Disease. In AD, processing of amyloid precursor protein (APP) by β- and γ-secretases produces the amyloid β-peptide (Aβ) with 40–42 amino acids, which accumulate extracellularly, forming insoluble structures. In fact, Aβ is the prominent component of senile plaques found in brains of AD patients. Moreover, intracellular neurofibrillary tangles formed by phosphorylation of tau are also found, although not restricted to AD. Age is the main risk for AD, characterized by a global cognitive decline. Autosomal dominant familial forms are caused by mutations in APP, presenilin 1 (PS1), or presenilin 2 (PS2), mainly associated with the early onset of AD.

Aβ has been implicated in neuronal and vascular degeneration in brain regions susceptible to plaque formation, because it can cause toxicity to neurons and to endothelial cells. In a previous report, Xu et al. (83) have shown that both $A\beta_{25–35}$ and $A\beta_{1–40}$ induced apoptotic cell death in cerebral endothelial cells, as evaluated by increased oxidative stress, caspase activation, mitochondrial dysfunction, and DNA damage. In fact, Aβ-neurotoxicity has been associated with almost all cell death processes (apoptosis and necrosis), including mitochondrial dysfunction (due to complex IV inhibition) associated with impaired energy metabolism, increased generation of ROS (including formation of carbonyl groups, lipid peroxidation, and nucleic acid oxidation), disruption of Ca^{2+} homeostasis, excitotoxicity, altered synaptic plasticity, apoptotic features of cell death involving the mitochondria (e.g., cytochrome c release, and activation of caspases -2, -9, and -3), ER-stress (e.g., caspase-12 activation), and DNA fragmentation/condensation, activation of inflammatory processes, leading to proliferation of microglia and general loss of cell viability (e.g., reviewed in [10]). Under this perspective, we have shown that Aβ exacerbates the Ca^{2+}-induced opening of the PTP in brain mitochondria, without inducing the PTP per se (84). In addition, we have demonstrated that a functional mitochondria is required for Aβ-induced neurotoxicity, as investigated using ρ+ and ρ0 mitochondrial DNA depleted cells (85). Interestingly, several antioxidants (vitamin E, idebenone, and reduced glutathione), melatonin, and nicotine showed protective effects by maintaining the mitochondrial membrane potential, improving the activity of the respiratory complexes and the cellular energetic levels (85).

APP can be proteolytically cleaved by caspases -6, -8, and -9, generating a C-terminal peptide (unrelated with Aβ) that can induce cell death not only in AD but also in other neurodegenerative disorders. Recently, a novel protein, the presenilin-associated protein (PSAP), shown to interact with PS1 C-terminus, was reported to be a mitochondrial resident protein associated with a proapoptotic activity through the release of cytochrome c, caspase-3 activation, DNA fragmentation, and cleavage of PARP (86). Importantly, $A\beta_{1–40}$ neurotoxicity also requires NMDA excitotoxicity, as determined by the protective effects of memantine, an uncompetitive NMDA receptor antagonist, upon $A\beta_{1–40}$ injection in the hippocampus, an insult causing neuronal degeneration, pyknotic nuclei, and astrocytic and microglia activation in this cerebral area (87).

Insulin dysfunction has been suggested to play a role in AD because diabetes mellitus seems to increase the risk of AD (88). The insulin receptor was shown to be expressed in the hippocampus and in the cerebral cortex, two brain areas affected in AD, but its role in the brain is still unclear. Although insulin may regulate the phosphorylation of tau or interfere with the metabolism of APP, any beneficial effect of the hormone as a therapeutic intervention against AD is still questionable (88). Nevertheless, insulin has been suggested to play an antiapoptotic role by activating NF-kB, increasing the expression of Mn-SOD in a cell line (89) or by reducing the activation of caspase-3 through a mechanism dependent on PI3K/Akt signaling pathway in retinal neurons (90).

Huntington's Disease. HD is a genetic autosomic disease characterized by the expansion of CAG repeats in the gene that codes for huntingtin, a cytoplasmic protein of unknown function. Because the CAG triplet codes for glutamine, when mutated, the protein presents a polyglutamine tract at the N-terminus resulting from more than 38/39–55 CAG repeats (in adult-onset HD), while expansion of 70 repeats or more occur in juvenile cases, in contrast with normal individuals containing less than 35 CAG repeats. In fact, expansion of CAG repeats is the underlying cause of at least nine inherited human neurodegenerative disorders, including HD and spinocerebellar ataxias.

Polyglutamine fragments accumulate as aggregates in the cytoplasm or nucleus (nuclear inclusions), and induce neuronal cell death. However, the molecular mechanism of polyglutamine-induced cell death is still controversial. HD is characterized by abnormal involuntary movements and cognitive impairment, with a predominant dysfunction in the striatum (particularly the striatal spiny neurons) and cortex. A decrease in the activity of mitochondrial complex II has been observed in the brains of HD patients, and interestingly, systemic injection of 3-nitropropionic acid was shown to mimic the pathological hallmarks of HD in several animal models by inducing a preferential degeneration of the striatum. Such a lesion exhibited nuclear DNA fragmentation and strong expression of the proapoptotic protein Bax, suggesting the occurrence of apoptotic cell death at the site of the severe striatal lesion induced by 3-nitropropionic acid (91). This toxin was recently shown to induce striatal degeneration via activation (by phosphorylation) of the c-Jun N-terminal kinase, followed by its translocation to the nucleus and activation of the transcription factor c-Jun (92).

Apoptotic features, determined by a redistribution of cytochrome c and activation of caspase-9, were also observed in both HD patients and in the R6/2 transgenic model of HD at the endstage of the disease, correlated with severe neuropathological deficits (93). Both caspase and calpain cleavage sites were identified in huntingtin (94), leading to the formation of truncated, smaller fragments that accumulate in the nucleus, potentiating huntingtin cytotoxicity. Cleavage by calpains was shown to depend upon the size of the polyglutamine repeat tract (increased length correlated with increased susceptibility) and occurred independently of caspase cleavage. Nevertheless, in transgenic mouse models of HD, no signs of cell death by apoptosis or necrosis were observed in dying neurons exhibiting intranuclear inclusions, because organelle structure was preserved, without evidence of membrane blebbing, apoptotic bodies, or fragmentation of DNA (95).

In the YAC transgenic mouse model expressing full-length huntingtin, an increased susceptibility for NMDA receptor–induced neuronal death, associated with NR2B-subunits and caspase-3 activation, was observed upon intrastriatal injection of quinolinic acid (96), confirming the evidence that implicated the process of excitotoxicity in HD neuropathology. Interestingly, mitochondria isolated from lymphoblasts of HD patients or brain mitochondria obtained from transgenic mice expressing full-length huntingtin showed a decreased $\Delta\Psi_m$ and depolarized for lower Ca^{2+} concentrations, compared with the control (97). This effect could be accounted for by the identification of N-terminal mutant huntingtin on neuronal mitochondria, highly suggesting its direct effect on the organelle.

Normal huntingtin, on the other hand, has been described to be neuroprotective, by playing an anti-apoptotic role as a blocker of procaspase-9 processing, interfering with the apoptosome complex, downstream of cytochrome c release (98). However, a Ca^{2+}-dependent cleavage (by calpains) of wild-type huntingtin under stressed conditions (e.g., glutamate excitotoxicity or mitochondrial dysfunction resulting from complex II inhibition) was reported to reduce the neuroprotective activity of wild-type huntingtin (99).

CONCLUSION

In recent years the mitochondria have assumed a great importance by clarifying the link between different signaling molecules (e.g., cytosolic Ca^{2+}) and the commitment to cell death. Mitochondria regulate the death execution phase, marking the point of no return in necrosis and apoptosis. Therefore we need to know more about the less clarified upstream mitochondrial pathways that can be involved in the initiation phase of human neurodegenerative disorders. This can be accomplished by studying, for example, the caspase-2–dependent initiation of apoptosis, which seems to "use" the mitochondria as an amplification route, and the intricate pathways mediated by the activation of the receptors for neurotrophins, which, when disrupted, may abrogate the mechanisms of neuronal survival. Furthermore, because damaged mitochondria can accumulate in aging as a result of deficient autophagy (100), it will be also important to identify the mechanisms involved in autophagy in neurons committed to die in various neurodegenerative disorders.

ACKNOWLEDGMENTS

The authors are grateful to Maria Teresa M. C. Oliveira (Center for Neuroscience and Cell Biology, University of Coimbra, Portugal) for helpful assistance with the preparation of Figs. 1 and 2.

REFERENCES

1. Frandsen, A. and Schousboe, A. 1993. Excitatory amino acid-mediated cytotoxicity and calcium homeostasis in cultured neurons. J. Neurochem. 60:1202–1211.
2. Tymianski, M., Charlton, M. P., Carlen, P. L., and Tator, C. H. 1993. Source specificity of early calcium neurotoxicity in cultured embryonic spinal neurons. J. Neurosci. 13:2085–2104.

3. Cebers, G., Zhivotovsky, B., Ankarcrona, M., and Liljequist, S. 1997. AMPA neurotoxicity in cultured cerebellar granule neurons: Mode of cell death. Brain Res. Bull. 43:393–403.
4. Carriedo, S. G., Sensi, S. L., Yin, H. Z., & Weiss, J. H. 2000. AMPA exposures induce mitochondrial Ca^{2+} overload and ROS generation in spinal motor neurons in vitro. J. Neurosci. 20:240–250.
5. Liu, W., Liu, R., Chun, J. T., Bi, R., Hoe, W., Schreiber, S. S., and Baudry, M. 2001. Kainate excitotoxicity in organotypic hippocampal slice cultures: Evidence for multiple apoptotic pathways. Brain Res. 916:239–248.
6. McCormack, J. G., Halestrap, A. P., and Denton, R. M. 1990. Role of calcium ions in regulation of mammalian intramitochondrial metabolism. Physiol. Rev. 70:391–425.
7. Kowaltowski, A. J., Castilho, R. F., and Vercesi, A. E. 1995. Ca^{2+}-induced mitochondrial membrane permeabilization: Role of coenzyme Q redox state. Am. J. Physiol. 269:141–147.
8. Halliwell, B. 1992. Reactive oxygen species and the central nervous system. J. Neurochem. 59:1609–1623.
9. Ankarcrona, M., Dypbukt, J. M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S. A., and Nicotera, P. 1995. Glutamate-induced neuronal death: A sucession of necrosis or apoptosis depending on mitochondrial function. Neuron 15:961–973.
10. Mattson, M. P. 2000. Apoptosis in neurodegenerative disorders. Nat. Rev. Mol. Cell Biol. 1:120–129.
11. Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. 1998. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 94:481–490.
12. Kim, T.-H., Zhao, Y., Barber, M. J., Kuharsky, D. K., and Yin, X.-M. 2000. Bid-induced cytochrome c release is mediated by a pathway independent of mitochondrial permeability transition pore and Bax. J. Biol. Chem. 275:39474–39481.
13. Martin, A. G. and Fearnhead, H. O. 2002. Apocytochrome c blocks caspase-9 activation and Bax-induced apoptosis. J. Biol. Chem. 277:50834–50841.
14. Liu, X., Zou, H., Slaughter, C., and Wang, X. 1997. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 89:175–184.
15. Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D. R., Aebersold, R., Siderovski, D. P., Penninger, J. M., and Kroemer, G. 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441–446.
16. Candé, C., Cecconi, F., Dessen, P., and Kroemer, G. 2002. Apoptosis-inducing factor (AIF): Key to the conserved caspase-independent pathways of cell death? J. Cell Sci. 115:4727–4734.
17. Li, L. Y., Luo, X., and Wang, X. 2001. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature 412:95–99.
18. Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J., and Vaux, D. L. 2000. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 102:43–53.
19. Du, C., Fang, M., Li, Y., Li, L., and Wang, X. 2000. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 102:33–42.
20. MacFarlane, M., Merrison, W., Bratton, S. B., and Cohen, G. M. 2002. Proteasome-mediated degradation of Smac during apoptosis: XIAP promotes Smac ubiquitation in vitro. J. Biol. Chem. 277:36611–36616.
21. Springs, S. L., Diavolitsis, V. M., Goodhouse, J., and McLendon, G. L. 2002. The kinetics of translocation of Smac/Diablo from the mitochondria to the cytosol in HeLa cells. J. Biol. Chem. 277:45715–45718.
22. Finucane, D. M., Waterhouse, N. J., Amarante-Mendes, G. P., Cotter, T. G., and Green, D. R. 1999. Collapse of the inner mitochondrial transmembrane potential is not required for apoptosis of HL60 cells. Exp. Cell Res. 251:166–174.
23. Heiskanen, K. M., Bhat, M. B., Wang, H.-W., Ma, J., and Nieminen, A.-L. 1999. Mitochondrial depolarization accompanies cytochrome c release during apoptosis in PC6 cells. J. Biol. Chem. 274:5654–5658.
24. Prehn, J. H. M., Jordán, J., Ghadge, G. D., Preis, E., Galindo, M. F., Roos, R. P., Krieglstein, J., and Miller, R. J. 1997. Ca^{2+} and reactive oxygen species in staurosporine-induced neuronal apoptosis. J. Neurochem. 68:1679–1685.
25. Rego, A. C., Vesce, S., and Nicholls, D. G. 2001. The mechanism of mitochondrial membrane potential retention following release of cytochrome c in apoptotic GT1–7 neural cells. Cell Death Differ. 8:995–1003.
26. Lassus, P., Opitz-Araya, X., and Lazebnik, Y. 2002. Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. Science 297:1352–1354.
27. Kumar, S. and Vaux, D. L. 2002. A Cinderella caspase takes center stage. Science 297:1290–1291.
28. Lemasters, J. J., Qian, T., Elmore, S. P., Trost, L. C., Nishimura, Y., Herman, B., Bradham, C. A., Brenner, D. A., Nieminen, A. L. 1998. Confocal microscopy of the mitochondrial permeability transition in necrotic cell killing, apoptosis and autophagy. Biofactors 8:283–285.
29. Uchiyama, Y. 2001. Autophagy cell death and its execution by lysosomal cathepsins. Arch. Histol. Cytol. 64:233–246.
30. Pastorino, J. G., Tafani, M., Rothman, R. J., Marcineviciute, A., Hoek, J. B., and Farber, J. L. 1999. Functional consequences of the sustained or transient activation by Bax of the mitochondrial permeability transition pore. J. Biol. Chem. 274:31734–31739.
31. Eskes, R., Antonsson, B., Osen-Sand, A., Montessuit, S., Richter, C., Sadoul, R., Mazzei, G., Nichols, A., and Martinou, J.-C. 1998. Bax-induced cytochrome c release from mitochondria is independent of the permeability transition pore but highly dependent on Mg^{2+} ions. J. Cell Biol. 143:217–224.
32. Mikhailov, V., Mikhailova, M., Pulkrabek, D. J., Dong, Z., Venkatachalam, M. A., and Saikumar, P. 2001. Bcl-2 prevents Bax oligomerization in the mitochondrial outer membrane. J. Biol. Chem. 276:18361–18374.
33. Henshall, D. C., Araki, T., Schindler, C. K., Lan, J.-Q., Tiekoter, K. L., Taki, W., and Simon, R. P. 2002. Activation of Bcl-2-associated death protein and counter-response of Akt within cell populations during seizure-induced neuronal death. J. Neurosci. 22:8458–8465.
34. Springer, J. E., Azbill, R. D., Nottingham, S. A., and Kennedy, S. E. 2000. Calcineurin-mediated Bad dephosphorylation activates the caspase-3 apoptotic cascade in traumatic spinal cord injury. J. Neurosci. 20:7246–7251.
35. Yang, J., Liu, X., Bhalla, K, Kim, C. N., Ibrado, A. M., Cai, J., Peng, T.-I., Jones, D. P., and Wang, X. 1997. Prevention of apoptosis by bcl-2: Release of cytochrome c from mitochondria blocked. Science 275:1129–1132.
36. Wang, N. S., Unkila, M. T., Reineks, E. Z., and Distelhorst, C. W. 2001. Transient expression of wild-type or mitochondrially targeted Bcl-2 induces apoptosis, whereas transient expression of endoplasmic reticulum-targeted Bcl-2 is protective against Bax-induced cell death. J. Biol. Chem. 276:44117–44128.
37. Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., Örd, T., and Bredesen, D. E. 1993. Bcl-2 inhibition of neural death: Decreased generation of reactive oxygen species. Science 262:1274–1277.
38. Ellerby, L. M., Ellerby, H. M., Park, S. M., Holleran, A. L., Murphy, A. N., Fiskum, G., Kane, D. J., Testa, M. P., Kayalar, C., and Bredesen, D. E. 1996. Shift of the cellular oxidation-reduction potential in neural cells expressing bcl-2. J. Neurochem. 67:1259–1267.
39. Zhu, L., Ling, S., Yu, X.-D., Venkatesh, L. K., Subramanian, T., Chinnadurai, G., and Kuo, T. H. 1999. Modulation of mitochondrial Ca^{2+} homeostasis by Bcl-2. J. Biol. Chem. 274: 33267–33273.

40. Krohn, A. J., Wahlbrink, T., and Prehn, J. H. M. 1999. Mitochondrial depolarization is not required for neuronal apoptosis. J. Neurosci. 19:7394–7404.
41. Shimizu, S. and Tsujimoto, Y. 2000. Proapoptotic BH3-only Bcl-2 family members induce cytochrome c release, but not mitochondrial membrane potential loss, and do not directly modulate voltage-dependent anion channel activity. Proc. Natl. Acad. Sci. USA 97:577–582.
42. Rizzuto, R., Brini, M., Murgia, M., and Pozzan, T. 1993. Microdomains with high Ca^{2+} close to IP_3-sensitive channels that are sensed by neighboring mitochondria. Science 262: 744–747.
43. Arnaudeau, S., Kelley, W. L., Walsh, Jr. J. V., and Demaurex, N. 2001. Mitochondria recycle Ca^{2+} to the endoplasmic reticulum and prevent the depletion of neighboring endoplasmic reticulum regions. J. Biol. Chem. 276:29430–29439.
44. Rapizzi, E., Pinton, P., Szabadkai, G., Wieckowski, M. R., Vandecasteele, G., Baird, G., Tuft, R. A., Fogarty, K. E., and Rizzuto, R. 2002. Recombinant expression of the voltage-dependent anion channel enhances the transfer of Ca^{2+} microdomains to mitochondria. J. Cell Biol. 159:613–624.
45. Chen, L. and Gao, X. 2002. Neuronal apoptosis induced by endoplasmic reticulum stress. Neurochem. Res. 27:891–898.
46. Aoki, S., Su, Q., Li, H., Nishikawa, K., Ayukawa, K., Hara, Y., Namikawa, K., Kiryu-Seo, S., Kiyama, H., and Wada, K. 2002. Identification of an axotomy-induced glycosylated protein, AIGP1, possibly involved in cell death triggered by endoplasmic reticulum-golgi stress. J. Neurosci. 22:10751–10760.
47. Rao, R. V., Hermel, E., Castro-Obregon, S., del Rio, G., Ellerby, L. M., Ellerby, H. M., and Bredesen, D. E. 2001. Coupling endoplasmic reticulum stress to the cell death program. J. Biol. Chem. 276:33869–33874.
48. Ferri, K. F. and Kroemer, G. 2001. Organelle-specific initiation of cell death pathways. Nat. Cell Biol. 3:E255–E263.
49. Peng, T.-I. and Greenamyre, J. T. 1998. Privileged access to mitochondria of calcium influx through *N*-methyl-D-aspartate receptors. Mol. Pharmacol. 53:974–980.
50. Stout, A. K., Raphael, H. M., Kanterewicz, B. I., Klann, E., and Reynolds, I. J. 1998. Glutamate-induced neuron death requires mitochondrial calcium uptake. Nat. Neurosci. 1:366–373.
51. Ward, M. W., Rego, A. C., Frenguelli, B. G., and Nicholls, D. G. 2000. Mitochondrial membrane potential and glutamate excitotoxicity in cultured cerebellar granule cells. J. Neurosci. 20:7208–7219.
52. Rego, A. C., Santos, M. S., and Oliveira, C. R. 2000. Glutamate-mediated inhibition of oxidative phosphorylation in cultured retinal cells. Neurochem. Int. 36:159–166.
53. Castilho, R. F., Hansson, O., Ward, M. W., Budd, S. L., and Nicholls, D. G. 1998. Mitochondrial control of acute glutamate excitotoxicity in cultured cerebellar granule cells. J. Neurosci. 18: 10277–10286.
54. Alano, C. C., Beutner, G., Dirksen, R. T., Gross, R. A., and Sheu, S. S. 2002. Mitochondrial permeability transition and calcium dynamics in striatal neurons upon intense NMDA receptor activation. J. Neurochem. 80:531–538.
55. Atlante, A., Calissano, P., Bobba, A., Azzariti, A., Marra, E., and Passarella, S. 2000. Cytochrome c is released from mitochondria in a reactive oxygen species (ROS)-dependent fashion and can operate as a ROS scavenger and as a respiratory substrate in cerebellar neurons undergoing excitotoxic death. J. Biol. Chem. 275:37159–37166.
56. Luetjens, C. M., Bui, N. T., Sengpiel, B., Münstermann, G., Poppe, M., Krohn, A. J., Bauerbach, E., Krieglstein, J., and Prehn, J. H. M. 2000. Delayed mitochondrial dysfunction in excitotoxic neuron death: Cytochrome c release and a secondary increase in superoxide production. J. Neurosci. 20:5715–5723.
57. Tenneti, L. and Lipton, S. A. 2000. Involvement of activated caspase-3-like proteases in *N*-methyl-D-aspartate-induced apoptosis in cerebrocortical neurons. J. Neurochem. 74:134–142.
58. Yu, S. W., Wang, H., Poitras, M. F., Coombs, C., Bowers, W. J., Federoff, H. J., Poirier, G. G., Dawson, T. M., and Dawson, V. L. 2002. Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297: 259–263.
59. Rego, A. C., Ward, M. W., and Nicholls, D. G. 2001. Mitochondria control AMPA/kainate receptor-induced cytoplasmic calcium deregulation in rat cerebellar granule cells. J. Neurosci. 21:1893–1901.
60. Kiedrowski, L. 1998. The difference between mechanisms of kainate and glutamate excitotoxicity in vitro: Osmotic lesion versus mitochondrial depolarization. Restor. Neurol. Neurosci. 12:71–79.
61. Larm, J. A., Cheung, N. S., and Beart, P. M. 1997. Apoptosis induced via AMPA-selective glutamate receptors in cultured murine cortical neurons. J. Neurochem. 69:617–622.
62. Rego, A. C., Monteiro, N. M., Silva, A. P., Gil, J., Malva, J. O., and Oliveira, C. R. 2003. Mitochondrial apoptotic cell death and moderate superoxide generation upon selective activation of non-desensitizing AMPA receptors in hippocampal cultures. J. Neurochem. (in press).
63. Itoh, T., Itoh, A., Horiuchi, K., and Pleasure, D. 1998. AMPA receptor-mediated excitotoxicity in human NT2-N neurons results from loss of intracellular Ca^{2+} homeostasis following marked elevation of intracellular Na^{+}. J. Neurochem. 71:112–124.
64. Turrens, J. F., Alexandre, A., Lehninger, A. L. 1985. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. Arch. Biochem. Biophys. 237:408–414.
65. Maciel, E. N., Vercesi, A. E., and Castilho, R. F. 2001. Oxidative stress in Ca(2+)-induced membrane permeability transition in brain mitochondria. J. Neurochem. 79:1237–1245.
66. Patel, M., Day, B. J., Crapo, J. D., Fridovich, I., and McNamara, J. O. 1996. Requirement for superoxide in excitotoxic cell death. Neuron 16:345–355.
67. Bindokas, V. P., Jordán, J., Lee, C. C., and Miller, R. J. 1996. Superoxide production in rat hippocampal neurons: Selective imaging with hydroethidine. J. Neurosci. 16:1324–1336.
68. Cai, J. and Jones, D. P. 1998. Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome c loss. J. Biol. Chem. 273:11401–11404.
69. Sugawara, T., Noshita, N., Lewén, A., Gasche, Y., Ferrand-Drake, M., Fujimura, M., Morita-Fujimura, Y., and Chan, P. H. 2002. Overexpression of copper/zinc superoxide dismutase in transgenic rats protects vulnerable neurons against ischemic damage by blocking the mitochondrial pathway of caspase activation. J. Neurosci. 22:209–217.
70. Greenlund, L. J., Deckwerth, T. L., and Johnson, E. M., Jr. 1995. Superoxide dismutase delays neuronal apoptosis: A role for reactive oxygen species in programmed neuronal death. Neuron 14:303–315.
71. Takeyama, N., Miki, S., Hirakawa, A., and Tanaka, T. 2002. Role of the mitochondrial permeability transition and cytochrome c release in hydrogen peroxide-induced apoptosis. Exp. Cell Res. 274:16–24.
72. Kruman, I., Guo, Q., and Mattson, M. P. 1998. Calcium and reactive oxygen species mediate staurosporine-induced mitochondrial dysfunction and apoptosis in PC12 cells. J. Neurosci. Res. 51:293–308.
73. Forrest, V. J., Kang, Y.-H., McClain, D. E., Robinson, D. H., and Ramakrishnan, N. 1994. Oxidative stress-induced apoptosis prevented by trolox. Free Radic. Biol. Med. 16:675–684.
74. Krohn, A. J., Preis, E., and Prehn, J. H. M. 1998. Staurosporine-induced apoptosis of cultured rat hippocampal neurons involves caspase-1-like proteases as upstream initiators and increased production of superoxide as a main downstream effector. J. Neurosci. 18:8166–8197.
75. Ahlemeyer, B. and Krieglstein, J. 2000. Inhibition of glutathione depletion by retinoic acid and tocopherol protects cultured

neurons from staurosporine-induced oxidative stress and apoptosis. Neurochem. Int. 36:1–15.
76. Welch, W. J. and Gambetti, P. 1998. Chaperoning brain diseases. Nature 392:23–24.
77. Hyun, D.-H., Lee, M., Hattori, N., Kubo, S.-I., Mizuno, Y., Halliwell, B., and Jenner, P. 2002. Effect of wild-type or mutant parkin on oxidative damage, nitric oxide, antioxidant defenses and the proteasome. J. Biol. Chem. 277:28572–28577.
78. Lee, H. J., Shin, S. Y., Choi, C., Lee, Y. H., Lee, S. J. 2002. Formation and removal of alpha-synuclein aggregates in cells exposed to mitochondrial inhibitors. J. Biol. Chem. 277:5411–5417.
79. Sherer, T. B., Betarbet, R., Stout, A. K., Lund, S., Baptista, M., Panov, A. V., Cookson, M. R., and Greenamyre, J. T. 2002. An in vitro model of Parkinson's disease: Linking mitochondrial impairment to altered alpha-synuclein metabolism and oxidative damage. J. Neurosci. 22:7006–7015.
80. Kitamura, Y., Inden, M., Miyamura, A., Kakimura, J., Taniguchi, T., and Shimohama, S. 2002. Possible involvement of both mitochondria-and endoplasmic reticulum-dependent caspase pathways in rotenone-induced apoptosis in human neuroblastoma SH-SY5Y cells. Neurosci. Lett. 333:25–28.
81. da Costa, C. A., Paitel, E., Vincent, B., and Checler, F. 2002. α-Synuclein lowers p53-dependent apoptotic response of neuronal cells: Abolishment by 6-hydroxydopamine and implication for Parkinson's disease. J. Biol. Chem. 277:50980–50984.
82. Lee, M., Hyun, D., Halliwell, B., and Jenner, P. 2001. Effect of the overexpression of wild-type or mutant alpha-synuclein on cell susceptibility to insult. J. Neurochem. 76:998–1002.
83. Xu, J., Chen, S., Ku, G., Ahmed, S. H., Xu, J., Chen, H., and Hsu, C. Y. 2001. Amyloid beta peptide-induced cerebral endothelial cell death involves mitochondrial dysfunction and caspase activation. J. Cereb. Blood Flow Metab. 21:702–710.
84. Moreira, P. I., Santos, M. S., Moreno, A., Rego, A. C., and Oliveira, C. 2002. Effect of amyloid beta-peptide on permeability transition pore: a comparative study. J. Neurosci. Res. 69:257–267.
85. Cardoso, S. M., Santos, S., Swerdlow, R. H., and Oliveira, C. R. 2001. Functional mitochondria are required for amyloid β-mediated neurotoxicity. FASEB J. 15:1439–1441.
86. Xu, X., Shi, Y., Gao, W., Mao, G., Zhao, G., Agrawal, S., Chisolm, G. M., Sui, D., and Cui, M.-Z. 2002. The novel presenilin-1-associated protein is a proapoptotic mitochondrial protein. J. Biol. Chem. 277:48913–48922.
87. Miguel-Hidalgo, J. J., Alvarez, X. A., Cacabelos, R., and Quack, G. 2002. Neuroprotection by memantine against neurodegeneration induced by beta-amyloid (1–40). Brain Res. 958: 210–221.
88. Gasparini, L., Netzer, W. J., Greengard, P., and Xu, H. 2002. Does insulin dysfunction play a role in Alzheimer's disease? Trends Pharmacol. Sci. 23:288–293.
89. Bertrand, F., Desbois-Mouthon, C., Cadoret, A., Prunier, C., Robin, H., Capeau, J., Atfi, A., and Cherqui, G. 1999. Insulin antiapoptotic signaling involves insulin activation of the nuclear factor kB-dependent survival genes encoding tumor necrosis factor receptor–associated factor 2 and manganese superoxide dismutase. J. Biol. Chem. 274:30596–30602.
90. Barber, A. J., Nakamura, M., Wolpert, E. B., Reiter, C. E. N., Seigel, G. M., Antonetti, D. A., and Gardner, T. W. 2001. Insulin rescues retinal neurons from apoptosis by a phosphatidylinositol-3-kinase/Akt-mediated mechanism that reduces the activation of caspase-3. J. Biol. Chem. 276:32814–32821.
91. Vis, J. C., Verbeek, M. M., de Waal, R. M., ten Donkelaar, H. J., and Kremer, B. 2001. The mitochondrial toxin 3-nitropropionic acid induces differential expression patterns of apoptosis-related markers in rat striatum. Neuropathol. Appl. Neurobiol. 27:68–76.
92. Garcia, M., Vanhoutte, P., Pages, C., Besson, M. J., Brouillet, E., and Caboche, J. 2002. The mitochondrial toxin 3-nitropropionic acid induces striatal neurodegeneration via a c-Jun N-terminal kinase/c-Jun module. J. Neurosci. 22:2174–2184.
93. Kiechle, T., Dedeoglu, A., Kubilus, J., Kowall, N. W., Beal, M. F., Friedlander, R., Hersch, S. M., and Ferrante, R. J. 2002. Cytochrome c and caspase-9 expression in Huntington's disease. Neuromol. Med. 1:183–195.
94. Gafni, J. and Ellerby, L. M. 2002. Calpain activation in Huntington's disease. J. Neurosci. 22:4842–4849.
95. Turmaine, M., Raza, A., Mahal, A., Mangiarini, L., Bates, G. P., and Davies, S. W. 2000. Nonapoptotic neurodegeneration in a transgenic mouse model of Huntington's disease. Proc. Natl. Acad. Sci. 97:8093–8097.
96. Zeron, M. M., Hansson, O., Chen, N., Wellington, C. L., Leavitt, B. R., Brundin, P., Hayden, M. R., and Raymond, L. A. 2002. Increased sensitivity to *N*-methyl-D-aspartate receptor-mediated excitotoxicity in a mouse model of Huntington's disease. Neuron 33:849–860.
97. Panov, A. V., Gutekunst, C. A., Leavitt, B. R., Hayden, M. R., Burke, J. R., Strittmatter, W. J., and Greenamyre, J. T. 2002. Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines. Nat. Neurosci. 5:731–736.
98. Rigamonti, D., Sipione, S., Goffredo, D., Zuccato, C., Fossale, E., and Cattaneo, E. 2001. Huntingtin's neuroprotective activity occurs via inhibition of procaspase-9 processing. J. Biol. Chem. 276:14545–14548.
99. Goffredo, D., Rigamonti, D., Tartari, M., De Micheli, A., Verderio, C., Matteoli, M., Zuccato, C., and Cattaneo, E. 2002. Calcium-dependent cleavage of endogenous wild-type huntingtin in primary cortical neurons. J. Biol. Chem. 277:39594–39598.
100. Brunk, U. T. and Terman, A. 2002. The mitochondrial-lysosomal axis theory of aging: Accumulation of damaged mitochondria as a result of imperfect autophagocytosis. Eur. J. Biochem. 269: 1996–2002.