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Deadly Conversations: Nuclear-Mitochondrial Cross-Talk

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Neuronal damage following stroke or neurodegenerative diseases is thought to stem in part from overexcitation of *N*-methyl-D-aspartate (NMDA) receptors by glutamate. NMDA receptors triggered neurotoxicity is mediated in large part by activation of neuronal nitric oxide synthase (nNOS) and production of nitric oxide (NO). Simultaneous production of superoxide anion in mitochondria provides a permissive environment for the formation of peroxynitrite (ONOO−). Peroxynitrite damages DNA leading to strand breaks and activation of poly(ADP-ribose) polymerase-1 (PARP-1). This signal cascade plays a key role in NMDA excitotoxicity, and experimental models of stroke and Parkinson's disease. The mechanisms of PARP-1-mediated neuronal death are just being revealed. While decrements in ATP and NAD are readily observed following PARP activation, it is not yet clear whether loss of ATP and NAD contribute to the neuronal death cascade or are simply a biochemical marker for PARP-1 activation. Apoptosis-inducing factor (AIF) is normally localized to mitochondria but following PARP-1 activation, AIF translocates to the nucleus triggering chromatin condensation, DNA fragmentation and nuclear shrinkage. Additionally, phosphatidylserine is exposed and at a later time point cytochrome c is released and caspase-3 is activated. In the setting of excitotoxic neuronal death, AIF toxicity is caspase independent. These observations are consistent with reports of biochemical features of apoptosis in neuronal injury models but modest to no protection by caspase inhibitors. It is likely that AIF is the effector of the morphologic and biochemical events and is the commitment point to neuronal cell death, events that occur prior to caspase activation, thus accounting for the limited effects of caspase inhibitors. There exists significant cross talk between the nucleus and mitochondria, ultimately resulting in neuronal cell death. In exploiting this pathway for the development of new therapeutics, it will be important to block AIF translocation from the mitochondria to the nucleus without impairing important physiological functions of AIF in the mitochondria.

KEY WORDS: Ischemia; excitotoxicity; neurodegeneration; NMDA, nitric oxide; peroxynitrite; poly(ADP-ribose) polymerase; PARP-1.

INTRODUCTION

The extent and cost of neurologic disease is staggering. Fifty million Americans have a permanent, neurological disability that limits their daily activities. Chronic neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Lou Gehrig's disease (ALS) afflict over 6.5 million Americans. Every 53 s someone in the United States suffers a stroke, affecting over 3 million Americans each year with over 4.4 million stroke survivors who have significant disability. Clearly new strategies need to be developed to treat these patients. Basic fundamental research in cell signaling and biochemistry have begun to identify the key elements of the brain and nervous system that mediate neuronal injury. Identifying these signaling molecules would open the door to new clinical opportunities and have the potential to impact millions of lives.

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NEUROTOXICITY

Many pathways have been proposed for neuronal damage in stroke and neurodegenerative diseases including extrinsic and intrinsic apoptotic programs and excitotoxicity. Glutamate excitotoxicity is a common finding that is mediated by intracellular calcium, nitric oxide, and free radicals (Dirnagl *et al*., 1999; Kristian and Siesjo, 1998; Lipton, 1999). Glutamate initiates its actions postsynaptically by binding to four major types of receptors: metabotropic receptors, *N*-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate (AMPA) receptors, and kainate receptors (Mayer and Westbrook, 1987). NMDA receptor activation mediates, in large part, glutamate excitotoxicity and neuronal damage. Glutamate-stimulated NMDA receptors flux calcium and activate a variety of intracellular calcium-dependent enzymes and processes, of which activation of neuronal NO synthase (nNOS) plays a prominent role (Samdani *et al*., 1997). Thus, overproduction of NO from excessive or inappropriate stimulation of nNOS appears to mediate a major component of excitotoxic damage although other reactive oxygen species are also generated in excitotoxic conditions.

REACTIVE OXYGEN SPECIES (ROS)

The inherent biochemical and physiological characteristics of the brain, including high lipid concentrations and energy requirements, make it particularly susceptible to free-radical-mediated insult. When oxygen-free radicals are generated in excess of a cells antioxidant capacity severe damage to cellular constituents including proteins, DNA, and lipids (Chan, 2001) can occur. The oxygen species that are typically linked to oxidative stress are superoxide anion, hydroxyl radical ($^{\cdot}$OH), hydrogen peroxide (H_2O_2) nitric oxide (NO), and peroxynitrite (ONOO$-$). In mitochondria generation of these species from molecular oxygen is a normal aspect of mammalian respiration, and over activation of the NMDA receptor results in the increased formation of reactive oxygen species (Chan, 2001). NMDA-receptor-mediated stimulation of phospholipase A_2 and the subsequent release of arachidonic acid, prostaglandins, leukotrienes, thromboxanes, and platelet-activating factor leads to a variety of toxic events including generation of oxygen-free radicals (Dirnagl *et al*., 1999). These processes can cause the neuron to digest itself by protein breakdown, free-radical formation, and lipid peroxidation. Under conditions of calcium elevation and energy failure, xanthine dehydrogenase is converted to xanthine oxidase, the activity of which results in superoxide anion formation (Chan, 2001). The superoxide anion is generated by multiple pathways and is often placed at the start of an oxidative stress cascade. The brain derives most of its energy exclusively from oxidative respiration through the mitochondrial electron transport chain. Mitochondria are located throughout the neuronal perikarya and its processes. During the production of ATP there is a small high-energy electron "leak" (1–3%) resulting in the generation of superoxide anion. Superoxide anion is constrained by membranes that it cannot cross and is retained within mitochondria. Superoxide dismutase (SOD) catalyzes the conversion of superoxide anion to H_2O_2, which is diffusible within and between cells (Chan, 2001). NO is synthesized on demand by the enzyme NOS from the essential amino acid, L-arginine (Dawson and Dawson, 1998). There are three NOS genes, neuronal NOS (nNOS), endothelial NOS, and immunologic NOS that were named by the tissue from which they were first cloned. NO is small, diffusible, membrane permeable and reactive. The biochemical reactions involving NO are not well characterized. Probably the most important oxidant involved in the genesis of neurotoxicity is peroxynitrite (ONOO$-$). Peroxynitrite is formed from the reaction of NO with superoxide anion. In vitro the rate of this reaction is three times faster than the rate of reaction of the enzyme, SOD, in catalyzing the dismutation of superoxide anion (Ischiropoulos and Beckman, 2003). Therefore, when present at appropriate concentrations, NO can effectively compete with SOD for superoxide anion. Although a simple molecule, peroxynitrite is chemically complex. It has the activity of hydroxyl radical and nitrogen dioxide radical, although it does not readily decompose into these entities. Peroxynitrite can also directly nitrate and hydroxylate aromatic rings on amino acid residues. It is also a potent oxidant that reacts readily with sulfhydryls, with zinc-thiolate, lipids, proteins, and DNA (Ischiropoulos and Beckman, 2003).

POLY(ADP-RIBOSE) POLYMERASE (PARP)

The discovery that inhibitors of poly (ADP-ribose) polymerase (PARP) are neuroprotective against NMDA and NO neurotoxicity initiated interest in PARP activity in the CNS (Eliasson *et al*., 1997; Endres *et al*., 1997; Zhang *et al*., 1994). PARP-1 is a member of a growing family of proteins that now includs 18 putative PARP proteins based on protein domain homology and enzymatic function. PARP-1 is the PARP responsible for large branch chain polymers and generates >95% of the poly(ADP-ribose) in a cell. The obligatory trigger of PARP-1 activation is DNA strand nicks and breaks, which can be induced by a

variety of environmental stimuli and free radical/oxidant attacks, including oxidants (hydrogen peroxide, hydroxyl radical, peroxynitrite), ionizing radiation, and genotoxic agents such as N-methyl-N′-nitro-*N*-nitrosoguanidine (MNNG). In response to DNA damage, PARP-1 becomes activated and, using NAD^+ as a substrate, it builds up polymers of adenosine diphosphate ribose (de Murcia and Menissier de Murcia, 1994; Lindahl *et al*., 1995). Poly(ADP-ribose) acceptors include histones, topoisomerases I and II, DNA polymerases, and DNA ligase 2, as well as PARP-1 itself. Poly-ADP-ribosylation might result in an inhibition of the activity of some of these enzymes. In the case of histones, poly-ADP-ribosylation stimulates chromatin relaxation. The physiological function of PARP-1 and poly (ADP-ribosylation) is still under heavy debate. From studies using pharmacological inhibitors of PARP-1, poly(ADP-ribosylation) has been suggested to regulate gene expression and gene amplification, cellular differentiation and malignant transformation, cellular division, and DNA replication, as well as apoptotic cell death (Chiarugi, 2002).

Pharmacologic inhibition of PARP-1 or genetic knockout of PARP-1 elicits cytoprotection in a variety of disease models including ischemia-reperfusion injury, diabetes, inflammatory-mediated injury, reactive oxygen species-induced injury, glutamate excitotoxicity, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injury (Eliasson *et al*., 1997; Endres *et al*., 1997; Mandir *et al*. 1999; Szabo and Dawson, 1998; Yu *et al*., 2002; Zhang *et al*. 1994). How PARP-1 activation kills neurons is not known. A cell suicide hypothesis was developed in the 1980s (Berger and Berger, 1986; Berger *et al*., 1983) and has been used to explain the action of PARP-1 in the CNS (Fig. 1). The suicide theory is based on the role of cellular NAD^+ to regulate an array of vital cellular processes. NAD^+ serves as a cofactor for glycolysis and the tricarboxylic acid cycle, thus providing ATP for most cellular processes (Hageman and Stierum, 2001). NAD^+ also serves as the precursor for NADP, which acts as a cofactor for the pentose shunt, for bioreductive synthetic pathways, and is involved in the maintenance of reduced glutathione pools (Hageman and Stierum, 2001). The observation that

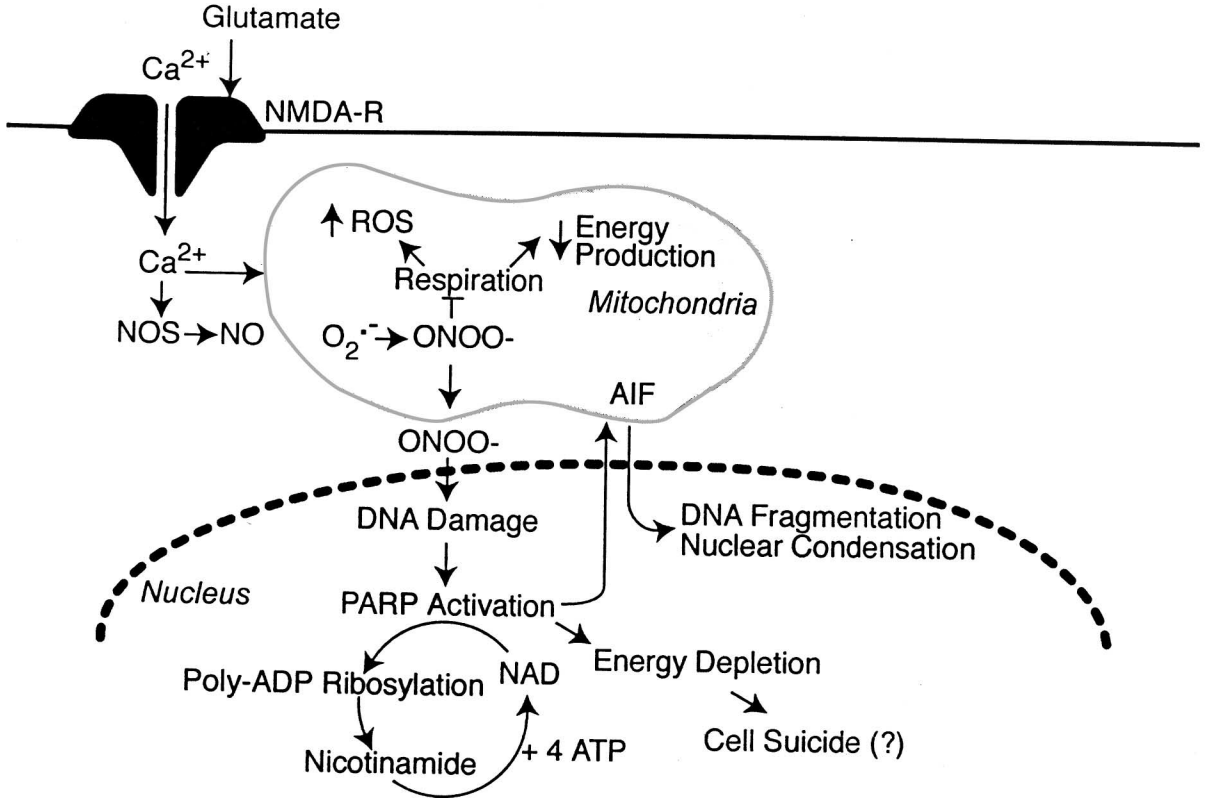

Fig. 1. Model of PARP-1-mediated excitotoxicity. NMDA receptor activation results in ROS and peroxynitrite (ONOO−) formation. ONOO- inhibits respiration leading to increased ROS and damages DNA-activating PARP-1. NAD is consumed to poly(ADP-ribosylate) proteins. PARP-1 activation results in AIF release from the mitochondria. Nuclear shrinkage and DNA fragmentation are observed when AIF enters the nucleus.

activation of PARP-1 can lead to massive NAD^+ utilization, and changes in the cellular NAD^+ and ATP levels led Berger and Okamoto to propose that consumption of NAD^+ due to DNA damage and activation of PARP-1 can affect cellular energetics and function ultimately leading to cell death due to excessive energy consumption (Berger and Berger, 1986; Berger *et al*., 1983). Studies in various cell and animal models have consistently observed depletion of NAD^+ and ATP that is blocked by PARP-1 inhibition or deletion. However, none of these studies address whether loss of cellular energetics is the cause of excitotoxic cell death or is simply a biomarker for PARP-1 activation. This is a difficult question to experimentally address. Addition of precursors such as creatine, can provide protection but these agents activate multiple mitochondrial and cellular pathways that may protect cells in a manner that is independent from the signal cascade triggered by PARP-1-mediated NAD^+ loss. In an experimental stroke model recent data indicate that preservation of energy stores in PARP-1 knockout mice is not the mechanism underlying the reduction in infarct volume (Goto *et al*., 2002). The time-course and severity of the apparent diffusion coefficient (ADC), an in vivo measure of cellular energy stores, is not altered in PARP-1 knockout brains compared with wild-type brains, despite the fact that the PARP-1 knockout animals had smaller infarct volumes compared with wild type animals (Goto *et al*., 2002). Thus, energy depletion alone might not be sufficient to mediate PARP-1-dependent cell death. Recently we identified a role for AIF as a downstream-signaling molecule in the PARP-1-dependent cell death but how PARP-1 triggers AIF release and cell death is not yet known (Fig. 1).

APOPTOSIS-INDUCING FACTOR (AIF)

Recent and emerging data indicate that AIF plays an important role in excitotoxic neuronal death. Mammalian AIF is a 67-kDa protein containing an *N*-terminal mitochondrial localization sequence and a large C-terminal with homology to bacterial oxidoreductases (Susin *et al*., 1999). AIF is evolutionarily conserved with homologs found in invertebrates, nematodes, fungi, and plants. AIF can stably bind FAD, which places AIF in the category of flavoproteins. AIF also displays NAD(P)H oxidase as well as monodehydroascorbate reductase activites (Miramar *et al*., 2001). The overall crystal structure of mature mouse AIF has been recently resolved at 2.0 Å resolution. AIF displays a glutathione-reductase-like fold, with an FAD-binding domain, an NADH-binding domain, and a C-terminal domain that bears a small AIF-specific insertion (509-559) not found in glutathione reductase. The amino acids interacting with FAD and NADH have been mapped precisely, and the mutants E313A and K176A have been shown to reduce FAD binding (Mate *et al*., 2002). Mutational analysis reveals that regardless of the presence or the absence of NAD(P)H and/or FAD (which is the essential prosthetic group of the oxidoreductase), AIF can induce nuclear apoptosis (Loeffler *et al*., 2001; Miramar *et al*., 2001). These data indicate that the oxidoreductase function of AIF is not required for its apoptogenic action.

The normal physiologic activity of AIF is not known. Recent data from the Harlequin mice in which expression of AIF 67 kDa is reduced 80% due to a proviral insertion into AIF genomic DNA suggests that AIF might participate in scavenging ROS (Klein *et al*., 2002). The putative redox reaction catalyzed by AIF in mitochondria is also not known. On the basis of its similarity to prokaryotic oxidoreductases, it is possible that AIF might interact with the cytochrome bc1 complex, which catalyzes the electron transfer from ubihydroquinone to cytochrome *c* in the mitochondrial respiratory chain (Mate *et al*., 2002). Theoretically, AIF could catalyze the reduction of cytochrome *c* in the presence of NADH in vitro (Miramar *et al*., 2001).

Following exposure of a cell to cytotoxic insults, AIF translocates to the cytosol and then the nucleus, where it induces peripheral chromatin condensation and high-molecular-weight (50 kb) DNA fragmentation. Translocation of AIF to the nucleus appears to be a general feature of apoptosis in mammalian cells (Cande *et al*., 2002) but whether it is a primary execution step or a secondary participatory step is dependent on the death signal and cell type. Confirmation of the death effector role for AIF when released from its normal mitochondrial compartment, are the observations resulting from forced cytosolic expression of AIF in the absence of an external death signal (Loeffler *et al*., 2001). The crystal structure of human AIF revealed the presence of a strong positive electrostatic potential at the AIF surface. AIF colocalizes with DNA at an early stage of nuclear morphological changes, as indicated by electron microscopy. The electrostatic interaction between AIF and DNA is independent of the DNA sequence. Structure-based mutagenesis showed that DNA-binding defective mutants of AIF fail to induce cell death (Ye *et al*., 2002). This suggests that DNA-binding by AIF is required for its apoptogenic function in the nuclear compartment. Two of the mutants that completely blocked the capacity of AIF to interact with DNA and to induce chromatin condensation (K255A, R265A, and K510A/K518A), still retained NADH oxidase activity (Ye *et al*., 2002), thus confirming that the oxidoreductase and apoptosis-inducing activities of AIF can be fully dissociated.

How AIF induces chromatin condensation and DNA fragmentation remains a mystery. There are several possible scenarios. AIF could itself have some cryptic nuclease activity that has not yet been observed. The interaction of AIF with DNA may increase the susceptibility of DNA to latent endogenous nucleases. AIF might recruit nucleases to induce partial chromatin fragmentation.

The mitochondrial-nuclear translocation of AIF is caspase-independent in some types of cell death (Cregan *et al*., 2002; Susin *et al*., 1999; Yu *et al*., 2002) as treatment with caspase inhibitors fails to block AIF translocation and cell death (Yu *et al*., 2002). Additionally, translocation of AIF can be observed in vitro in cells in which there is no caspase activation, owing to genetic deletion of Apaf-1, caspase-9 or caspase-3 (Cregan *et al*., 2002; Susin *et al*., 2000). Similar observations have been obtained in *Apaf*-1−/−, *caspase*-9−/− or *caspase*-3−/− embryoid bodies, in which AIF translocates during cavitation (Joza *et al*., 2001). Microinjection or transfection of *Apaf*-1−/−, *caspase*-9−/−, or *caspase*-3−/− cells with recombinant AIF protein or transient expression of AIF, also induces cell death without caspase activation. Features of classic apoptosis, such as phosphatidylserine exposure, partial chromatin condensation, and cellular shrinkage are still observed in these cells (Loeffler *et al*., 2001; Susin *et al*., 2000). In vitro, both purified natural AIF and recombinant AIF alter the structure of chromatin resulting in large-scale DNA fragmentation in purified nuclei. This "nuclear apoptosis" cannot be prevented by caspase inhibitors (Susin *et al*., 2000: Yu *et al*., 2002). While these data strongly support the notion that AIF acts as a caspase independent death effector, there also exists data for an interaction between AIF and the caspase cascade. Activated caspases (caspase-8 and caspase-2) and the caspase-activated protein *t*-Bid can trigger the release of AIF from mitochondria (Lassus *et al*., 2002; Robertson *et al*., 2002; Zamzami *et al*., 2000). In HeLa and Jurkat cell lines treated with staurosporine or actinomycin D, mitochondrial release of AIF is suppressed or delayed by caspase inhibitors (Arnoult *et al*., 2001). Genetic data obtained in *C. elegans* also suggest that AIF operates partially in a caspase-dependent fashion. Heat-shock inducible expression of Egl-1 causes the mitochondrial release of green fluorescent protein (GFP)-tagged AIF in all *C. elegans* wild-type embryos, but Ced- 3 loss-of-function mutants there is a >80% inhibition of AIF release (Wang *et al*., 2002). Transgenic expression of AIF and endonuclease G kills >60% of wild-type cells in *C. elegans*, but in *Ced*3-deficient animals cell death is reduced to 28%. These data suggest that, although AIF and endonuclease G can function in a caspase-independent fashion, AIF and endonuclease G are more efficient in a Ced-3-positive background (Wang *et al*., 2002). Taken together the published literature supports both caspase-independent or caspase-dependent mechanisms of AIF release from the mitochondria that are dependent on cell type and the lethal stimulus.

In the CNS AIF maybe be particularly important in mediating neurotoxicity due to both acute and chronic (neurodegenerative) diseases. Acute neurotoxicity can be induced by trauma, hypoglycemia, or transient ischemia. The translocation of AIF has been observed in several experimental models of neurotoxicity such as the death of photoreceptors induced by retinal detachment (Hisatomi *et al*., 2002), neuronal cell death induced in vivo by brain trauma (Zhang *et al*., 2002) and death of cortical neurons induced in vitro by exposure to heat-inactivated *Streptococcus pneumoniae* (Braun *et al*., 2001), hydrogen peroxide, peroxynitrite (Zhang *et al*., 2002), the topoisomerase I inhibitor camptothecin, infection with a p53-expressing adenovirus (Cregan *et al*., 2002), or the excitotoxin NMDA (Yu *et al*., 2002). In a model of neurotrauma, the translocation of AIF in selected brain areas could be correlated with genomic DNA degradation to ∼50 kb fragments (which is a hallmark of AIF-mediated nuclear apoptosis) (Zhang *et al*., 2002). We have shown that DNA-damage-induced AIF translocation and apoptosis depends on the presence of p53 and its transcriptional target Bax (Cregan *et al*., 2002). Under excitotoxic conditions we have shown that NMDA-induced release of AIF, is PARP-dependent. Microinjection of a neutralizing antibody recognizing a surface-exposed domain of AIF prevents cell death (Cregan *et al*., 2002; Yu *et al*., 2002) but caspase inhibition alone has no beneficial effect on cell survival (Braun *et al*., 2001; Yu *et al*., 2002; Zhang *et al*., 2002). Assuming that the anti-AIF antibody has no additional effects this suggests that AIF contributes to neurotoxicity. Taken together these data suggest that neurons are susceptible to both caspase-dependent and -independent death programs and that AIF can participate in both. In NMDA excitotoxicity the death program runs in a serial manner (Fig. 2). Blocking NOS, PARP, or AIF prevents the downstream events from occurring and prevents death. Caspase activation is a consequence of AIF release and does not contribute to cell death as inhibition of caspases does not produce cell survival. In neurotoxicity due to p53 activation following DNA damage parallel pathways are activated. The caspase pathway is primary but blocking this pathway merely delays cell death. A parallel p53 pathway also activates the AIF pathway. In order to elicit neuroprotection both parallel death programs, the caspase and AIF pathways, must be blocked. This type of parallel death machinery might be important in non-NMDA excitotoxicity elicited by AMPA or kainate.

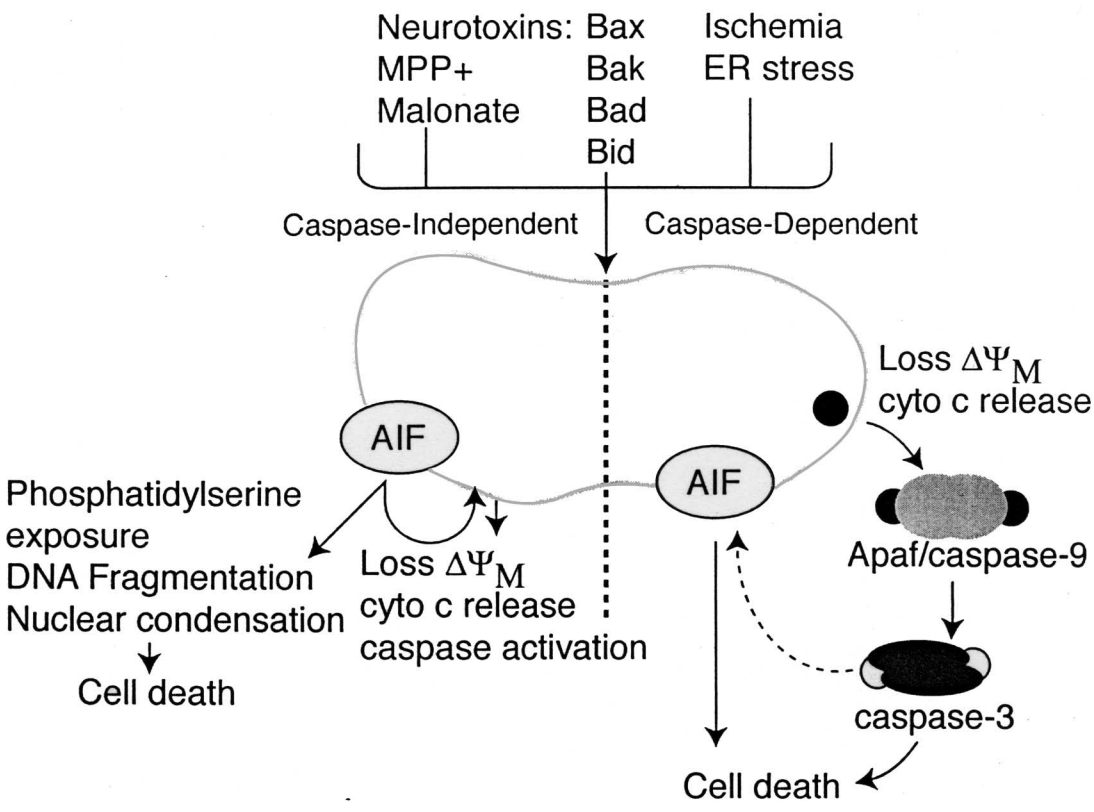

Fig. 2. AIF in caspase-independent and caspase-dependent cell death. Injury to the nervous system can elicit both caspase-dependent and -independent cell death that is both stimulus and cell type dependent. AIF can direct kill cells without caspase participation. In other death pathways caspases are active and are sufficient to kill the cell but can also recruit AIF to the death machinery. Additionally, some death stimuli, such as p53, activate AIF and caspase pathways simultaneously.

SUMMARY

In the CNS, injury is mediated by a carefully choreographed series of events initially triggered by calcium influx through the NMDA glutamate receptor activating nNOS and mitochondrial respiration (Fig. 3). The production of NO and superoxide anion result in the formation of peroxynitrite, which can diffuse from the mitochondria to damage various cellular constituents. In the nucleus the nicks in DNA induced by peroxynitrite activate the enzyme PARP-1 which consumes NAD in the process of generating PAR and modifying proteins with PAR. Activation of PARP-1 signals to the mitochondria and AIF is released. Translocation of AIF to the nucleus results in large-scale DNA fragmentation and nuclear shrinkage. This is likely the final commitment point to cell death. Genetic deletion studies and pharmacologic inhibition studies indicate that this pathway is critically important in several models of neurologic injury including models of stroke and Parkinson's disease. However, serial pathways may also be activated in some neurologic diseases. It is important to identify all pathways and order the sequence of events to better understand the signal cascades that result in neuronal death. It is the hope that a better understanding of these events will lead to identification of new target molecules to treat patients with neurologic disease.

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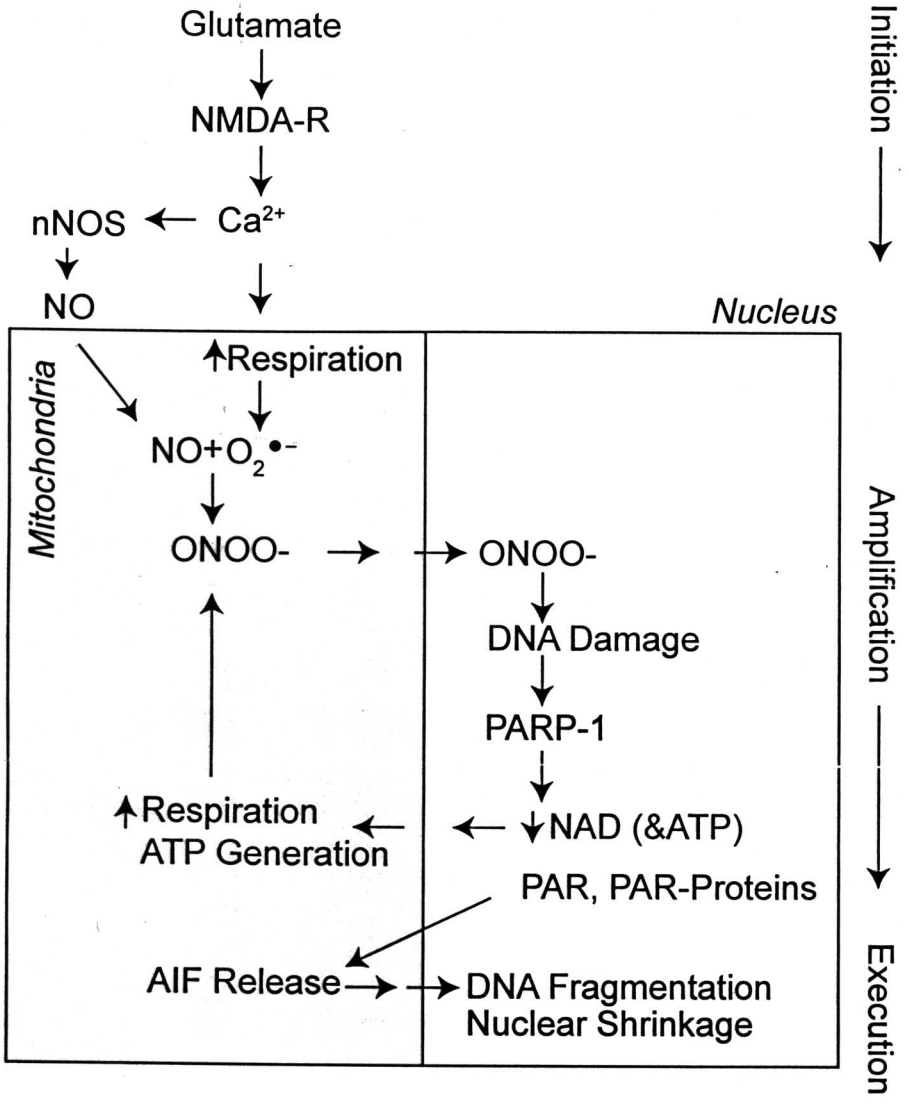

Fig. 3. Schematic of the ROS, PARP-1, AIF death program in excitotoxicity. In the initiation phase of neurotoxicity, activation of NMDA glutamate receptors leads to increased intracellular calcium that activates neuronal nitric oxide synthase (nNOS) producing nitric oxide (NO). Neuronal activation also results in increased oxidative phosphorylation and subsequently increased superoxide anion production in the mitochondria. Superoxide is not membrane permeable and resides largely in the mitochondria where it is generated. NO and superoxide anion react to form the potent oxidant, peroxynitrite. Peroxynitrite generation can trigger an amplification phase of neurotoxicity by attacking mitochondrial proteins in the electron transport chain including complex I and IV as well as the superoxide scavenging enzyme, manganese superoxide dismutase (MnSOD). This initiates a viscous cycle of peroxynitrite generation through sustained superoxide anion generation. Peroxynitrite is membrane permeable and can move to the nucleus triggering DNA strand breaks. Damaged DNA activates poly(ADP-ribose) polymerase (PARP) resulting in the synthesis of PAR polymers, ribosylation of proteins, and consumption of NAD and ATP. These PARP dependent events signal the mitochondria to release apoptosis inducing factor (AIF) that translocates to the nucleus. In the nucleus AIF triggers large scale DNA fragmentation and nuclear condensation. These nuclear changes in neurons are likely the final commitment and execution point in the neurotoxic cascade. Subsequent to these events cytochrome c is released and caspases are activated. Blocking these events does not prevent NO/PARP-dependent neurotoxicity but may be important in preparation of the corpse and the degradation of the cell.

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