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Delayed Neuronal Death in Ischemic Stroke: Molecular Pathways

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1. Introduction

Ischemic stroke is caused by a loss of blood flow and deficiency in glucose and oxygen to the brain. The lack of sufficient glucose and oxygen results in varying degrees of tissue damage and cell death following stroke. Reperfusion of blood flow after ischemia often compounds tissue damage that is sustained during the initial drop in local blood availability.

The size and position of the affected region depends on which vessel is occluded. A complete loss of blood flow is rare, as rich networks of nearby blood vessels often compensate for reduced flow. The centre of the ischemic region, the core, is characterized by acute and mostly necrotic cell death resulting from severe anoxia and hypoglycemia. The region enveloping the core is known as the penumbra, which experiences a milder ischemic insult. The penumbra should be targeted for treatment strategies; it is usually much larger than the core and has a longer window of opportunity during which neurons can be prevented from dying. Many studies elucidate the molecular pathways of delayed neuronal death. This chapter presents the pathways and strategies that have been investigated to date.

2. Events in stroke

There are major differences in the physiology and biochemistry of cell death between the core and penumbra, which suggests that different mechanisms of cell death are at work in these two regions.

2.1 Core of ischemic infarct

During a stroke, the ischemic core suffers a drop in energy. ATP levels in the core fall to a level only 15% of typical basal values within one or two minutes (Katsura et al., 1993; Lipton, 1999; Lipton & Whittingham, 1982; Martin et al., 1994) and do not recover by much after reperfusion (Sun et al., 1995). The core rapidly loses ion transporter functions and undergoes anoxic depolarization (Balestrino, 1995). Homeostasis of potassium, calcium, and sodium ions is lost (Harris & Symon, 1984). After reperfusion, extracellular K^+ typically returns to control levels for six hours and then stays slightly elevated above normal (Gido et al., 1997). There is some restoration of K^+ transporter functions, despite widespread cell

injury and cell death within the core. Other responses to ischemia-induced energy loss include reduction or a complete halting of protein synthesis due to translation initiation factor (IF) inactivation (White et al., 2000) and insufficient GTP for ribosomal function. Permanent absence of protein synthesis continuing beyond reperfusion results in necrotic cell death. Recovery of protein synthesis is necessary for cell survival. Necrosis in the core is accompanied by glutamate release and excitotoxic cell damage to neighbouring regions.

2.2 Penumbra of ischemic infarct

Blood flow within the penumbra can vary substantially, subjecting cells to a wide range of stresses. Ischemic injury within the penumbra is variable in whether it results in cell death and in which molecular mechanisms are involved. Many of these mechanisms induce cell death in a delayed manner in neurons, which allows them to be saved if some neuroprotection is provided. This delay allows for therapeutic treatment, since the majority of stroke patients present many hours after suffering a stroke.

Penumbral ischemia is milder than in the core; levels of ATP in the penumbra drop to an average of 50-70% of normal levels. Protein synthesis can be stalled following massive Ca^{2+} influx, which can inactivate eIF-2a by preventing activation of eIF-2 and guanine nucleotide exchange factor during the initiation of translation (Kumar et al., 2001). Protein synthesis resumes after reperfusion and has a role in determining the extent of delayed neuronal death.

2.3 Excitotoxicity

Penumbral cells are subject to excessive excitatory amino acid release from depolarized nearby cells in the ischemic core. Glutamate is the major excitatory neurotransmitter in the brain and the key mediator of intracellular communication, plasticity, growth and differentiation. The glutamate receptors implicated in excitotoxicity include the NMDA, AMPA, kainate, and other metabotropic glutamate receptors (Prass & Dirnagl, 1998). While present in synapses at micromolar concentrations, ischemia-induced depolarization causes a much larger release that triggers a chain reaction of depolarization and effects glutamate release in surrounding neurons (Paschen, 1996). The overstimulated neurons release Ca^{2+} into their cytosol, halting protein synthesis and activating cyclooxygenase-2 (COX-2), increased nitric oxide (NO) production, phospholipases, calpains, cathepsins, and calcineurin (Ferrer, 2006; White et al., 2000). Degradation of calpain substrates such as spectrin and eIF4G then follows (White et al., 2000), while cathepsin activation may increase lysosomal activity and lead to autophagic cell death (Yamashima et al., 1998). Membranes are degraded by hyperactivated phospholipases, which produce free arachidonic acid that is metabolized during reperfusion to produce peroxidative derivatives that then act as free radicals.

Excitotoxicity describes the damaging effects resulting from excessive excitatory neurotransmitter release. It is implicated in necrotic, apoptotic, and necroptotic cell death (Choi, 1996; Li et al., 2008).

2.4 Oxidative stress

Oxidative damage by free radical generation mediates cell damage in ischemia (Gilgun-Sherki et al., 2002). It is involved in excitotoxicity, apoptosis, autophagic cell death, and inflammation. Penumbral free radical levels increase during early ischemia, remain

elevated, and then rise during reperfusion due to a variety of metabolic and inflammatory mechanisms (Beckman et al., 1990; Chambers et al., 1985; Clemens et al., 1997; Kuehl et al., 1980; Zhu et al., 2004b). Arachidonic acid metabolites are a source of oxidative stress following reperfusion. Mitochondrial dysfunction, COX-2 activation, endothelial and neural production of NO, and conversion of xanthine dehydrogenase to xanthine oxidase play significant roles in producing free radicals.

Oxidative stress can cause lipid peroxidation, sulfhydryl oxidation, proteolysis, and destruction of nuclear material. Excessive free radicals can activate p53, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and activator protein-1, to drive their expression of pro-apoptotic genes. Free radicals and oxidative stress are involved in lysosomal dysfunction and autophagic cell death (Pivtoraiko et al., 2009). Free radicals can disrupt the electron transport chain in mitochondria, which results in autoxidation of flavoproteins and ubisemiquinone and an increase in superoxide generation (Zhu et al., 2004b).

2.5 Mitochondria-mediated death pathways

Neuronal mitochondria release cell-death factors and free radicals into the cytosol. Mitochondria have roles in apoptotic and necrosis-like cell death. ATP depletion, Ca^{2+} overload, and free radical damage causes the opening of mitochondrial permeability transition pores (Friberg & Wieloch, 2002) and releases cytochrome *c*, high temperature requirement protein A2 (HtrA2/Omi), second mitochondria-derived activator of caspases (Smac/DIABLO), apoptosis-inducing factor (AIF), and endonuclease G (EndoG).

The Bcl-2 family of proteins that regulate apoptosis includes both pro-apoptotic and anti-apoptotic proteins that counteract one another and regulate mitochondrial outer membrane permeability (MOMP) and release of mitochondrial apoptotic factors. After ischemic damage, Bcl-2 and Bcl-xL are inhibited, allowing Bax and Bak to act, which form channels in the mitochondria and release cytochrome *c*, HtrA2/Omi, and Smac/DIABLO and other cell-death-inducing factors.

2.6 Neurotrophins

Neurotrophins play a role in immediate protection of neurons and in long-term cellular remodeling and regeneration. Following ischemia, neurotrophic factors are upregulated (Ferrer et al., 1998; Takeda et al., 1993; Tsukahara et al., 1994), although local levels of brain-derived neurotrophic factor (BDNF) levels drop below baseline in vulnerable cell types (Kokaia et al., 1996). Other growth factors with neuroprotective effects include transforming growth factor-beta (TGF-β), acidic fibroblast growth factor (FGF1), and vascular endothelial growth factor (VEGF). Many mechanisms for the protective action of nerve growth factors that are induced or reduced post-ischemia have been proposed. FGF1 inhibits excitotoxicity by preventing or delaying the rise of Ca^{2+} during ischemia (Mitani et al., 1992). BDNF protects against excitotoxicity by preventing the decrease in protein kinase C that follows ischemia (Tremblay et al., 1999). The induction of BDNF is attributed to the reduction of free radicals (Mattson et al., 1995) and acts by upregulating antioxidant enzymes such as superoxide dismutases (SOD) and glutathione reductase. The function of BDNF depends on phosphorylating other cellular components (Dugan et al., 1997).

Maintenance of protein synthesis after ischemia is mediated by tyrosine kinase systems activated by neurotrophins or other growth factors. Lack of neurotrophic action in neurons

results in a failure to restore protein synthesis (Hu & Wieloch, 1994). TGF-β may provide neuroprotection in ischemia by moderating transcription factors and cell-death pathways. TGF-β controls the activation of mitogen-activated protein kinases (MAPK) (Friguls et al., 2002) and inhibits Bad and caspase-3 to reduce cell death (Buisson et al., 2003; Zhu et al., 2002). These effects may be mediated by NF-κB (Zhu et al., 2004a).

VEGF promotes angiogenesis, vascular permeability, and endothelial proliferation. VEGF is implicated in neurogenesis (Storkebaum et al., 2004). VEGF is up-regulated between six and 24 hours after stroke in the penumbra (Marti et al., 2000; Plate et al., 1999). In the penumbra, VEGF modulates the PI3K/Akt/NF-κB signalling pathway and inhibits caspase-3 activity to reduce apoptosis (Sun & Guo, 2005).

2.7 Heat shock proteins

Heat shock proteins (Hsp) are involved in proper protein folding and are expressed following heat and oxidative stresses. During the first minutes of stroke, Hsp70 and Hsp90 mRNA expression rises and persists in the penumbra (Ikeda et al., 1994; Kawagoe et al., 1992; Kinouchi et al., 1993; Woodburn et al., 1993), with upregulated protein expression following a few hours later. Cell survival is positively correlated with Hsp70 production, since overexpression of Hsp70 protects against infarction in rats (Mestril et al., 1996). Lower or reduced expression of Hsp70 positively correlates with neuronal death.

Expression of Hsps inhibits the activation of the transcription factor NF-κB (Schell et al., 2005), which primarily serves a detrimental function in ischemia. Hsp70 inhibits apoptosis through interacting with key proteins in various cell-death pathways. Hsp70 prevents activation of caspase-8 and caspase-9 (Matsumori et al., 2006). Hsp70 protects cells after caspase-3 activation by blocking activation of phospholipase A-2 in the cell nucleus (Jaattela et al., 1998). Hsps may work by preserving proper protein conformation in neurons suffering ischemia (Lipton, 1999).

Ubiquitin decreases after ischemia. Expression then recovers except in vulnerable neurons destined to die (Deshpande et al., 1992; Magnusson & Wieloch, 1989). This phenomenon may be involved in cell damage, possibly by allowing the accumulation of denatured proteins (Lipton, 1999).

3. Modes of neuronal cell death

Mechanisms of cell death vary along a continuum between regulated, programmed cell death and unregulated, necrotic cell death. One on end of the spectrum, apoptotic cell death is tightly regulated and normally involved in tissue maintenance. Necrosis, at the opposite end of the spectrum, is unregulated and results from injury. Between these extremes are pathways with varying semblance to either necrosis or apoptosis, including necrosis-like cell death, necroptosis, and autophagic cell death.

3.1 Necrosis

Classical necrosis lacks regulation, order, and energy dependence. It is caused by physical or chemical insult. It is characterized by an acute loss of osmotic homeostasis and an early decline in plasma membrane integrity and ATP levels, resulting in burst cells and inflammation from the scattered cell contents. DNA cleavage occurs late in cell death through a mechanism dependent on serine proteases (Bicknell & Cohen, 1995; Dong et al.,

1997). DNA fragments are of random size. This form of necrotic cell death is present during a stroke; it is found within the ischemic core during severe acute ischemic damage.

3.2 Autophagy

Autophagy is a regulated catabolic process involving the degradation of a cell's own cytoplasmic macromolecules and organelles through digestion by the lysosomal system. Autophagy can be triggered by defective cell machinery. Through the formation of autophagolysosomes, a cell is capable of degrading the constituents, effectively recycling macronutrients and reducing the cell's metabolic requirements. The role of autophagy in cell homeostasis is undisputed. Autophagocytosis as a unique mechanism of programmed cell death is a controversial concept. There is evidence that autophagy is a separate mechanism of cell death and not merely an adaptive response to nutrient limitation (Cho & Toledo-Pereyra, 2008). It is unclear if the observed autophagic processes and mechanisms associated with cell death are the effectors of cell death or merely an overshoot of their initially beneficial intentions.

The autophagic cell death process is distinct from apoptosis and necrosis; it is characterized by autophagic degradation of cellular components prior to nuclear destruction (Bursch et al., 2000a; Schwartz et al., 1993). The most representative morphological feature is the formation of numerous autophagosomes in the cytosol with a condensed nucleus (Bursch et al., 2000b). Evidence suggests that autophagy contributes to the neuronal degeneration following cerebral ischemia. Autophagy occurs in both neonatal and adult mouse cortices and hippocampi after ischemic injury. Increased autophagosomal marker LC3-II levels are detected as early as 8 h after ischemia; more pronunciation occurs at 24 h and 72 h after hypoxic ischemia (Koike et al., 2008; Zhu et al., 2005). Damaged neurons show features of autophagic cell death, such as increased lysosomal cysteine proteinases, formation of cytoplasmic autophagic vacuoles, and the induction of GFP-LC3 immunofluorescence, during cerebral hypoxia or ischemia in adult mice (Adhami et al., 2006; Nitatori et al., 1995). Inhibition of autophagy provides neuroprotection in situations where most other pharmacological treatments are ineffective (Puyal & Clarke, 2009).

3.2.1 Autophagy pathways

A pathway for autophagous cell death has been proposed that relies on the runaway activation of beclin 1. Beclin 1 is a primary inducer of autophagy and the first identified mammalian autophagy gene product (Aita et al., 1999). Beclin 1 was originally isolated as a Bcl-2-interacting protein (Liang et al., 1999). Bcl-2 inhibits beclin 1 and beclin-1-dependent autophagy in yeast and mammalian cells. Beclin 1 mutants that cannot bind to Bcl-2 induce more autophagy (Pattingre et al., 2005). The pharmacological BH3 mimetic ABT-737 can inhibit the interaction between beclin 1 and Bcl-2 or Bcl-xL and also stimulate autophagy (Maiuri et al., 2007a; Maiuri et al., 2007b). Beclin 1 is regulated through binding with Bcl-2 proteins. Bcl-2 downregulation may result in excessive autophagy causing cell death. Autophagy regulation through Bcl-2 is attributed to its expression at the ER membrane, suggesting that signalling events originating from the ER are crucial for autophagy (Rodriguez et al., 2011). ER stress triggers autophagy; this is regulated by UPR stress sensors (Kouroku et al., 2007; Ogata et al., 2006). Stimuli that increase cytosolic calcium can activate ER stress and autophagy, which can be blocked by Bcl-2 (Hoyer-Hansen et al., 2007). ER and

oxidative stresses, which are common in cerebral ischemia, are critical triggers of autophagy in neurons.

BH3-only proteins regulate autophagy under different settings, possibly by disrupting the interaction between beclin 1 and Bcl-2 or Bcl-xL via their BH3 domains (Bellot et al., 2009; Maiuri et al., 2007a). Prolonged expression or acute overexpression of BNIP3 beyond an autophagic survival threshold may result in autophagic cell death. Prolonged exposure to hypoxia of several apoptosis-competent cancer lines induces autophagy and cell death in a BNIP3-dependent manner. Beclin 1 liberation from Bcl-2 or Bcl-xL may be one of the mechanisms through which BH3-only members promote autophagy (Azad et al., 2008; Chinnadurai et al., 2008). BNIP3 may induce autophagy as a consequence of mitochondrial injury, as a loss of MPT induces autophagy (Elmore et al., 2001). Our lab has found a unique caspase-independent cell-death pathway that features the mitochondrial localization of BNIP3 followed by EndoG and AIF release from mitochondria and translocation into the nuclei, which results in cell death. Autophagy may play a part in this pathway by affecting mitochondrial stabilization or acting as a parallel cell-death-inducing pathway. Beclin 1 levels positively correlate with BNIP3 expression following ischemia. The increase in both proteins is accompanied by increased autophagic cell death that is inhibited by the autophagy inhibitor 3-methyladenine and by knockdown of BNIP3 with miRNA.

Autophagy and apoptosis can be triggered by upstream signals, often resulting in a mixed phenotype of both cell-death patterns. Neurons can switch between responses in a mutually exclusive manner. Both mechanisms are capable of inhibiting the other. Caspase inhibitors may arrest apoptosis but also promote autophagic cell death (Yu et al., 2004). Calpain-mediated cleavage of Beclin 1 can switch autophagy to apoptosis (Yousefi et al., 2006). Pathways linking the apoptotic and autophagic machineries have been deciphered at the molecular level (Maiuri et al., 2007c; Rubinsztein et al., 2005).

3.3 Apoptosis

Apoptosis is involved in cell development, differentiation, proliferation, homoeostasis, regulation, immune function, and removal of defective and harmful cells. In stroke, it is a mechanism of delayed neuronal death in response to ischemic injury. Key apoptotic proteins are activated and upregulated after cerebral ischemia, while inhibition of these proteins protects neurons from death (Chen et al., 1998).

Regulated apoptotic pathways activate cascades leading to cell suicide without the leakage of harmful cell contents. Main players in regulation include proteins from the Bcl-2 family, Smac/DIABLO (Du et al., 2000; Verhagen et al., 2000), HtrA2/Omi (Suzuki et al., 2001) and apoptotic protease-activating factor (Apaf-1) (Manfredi & Beal, 2000; Tatton & Olanow, 1999; Yuan & Yankner, 2000). Typical hallmarks of apoptosis include cell shrinkage and rounding, pyknosis and karyorhexis with DNA laddering on gel electrophoresis, membrane blebbing, and gradual disintegration of the cell into membrane-enclosed apoptotic bodies (Choi, 1996; Love, 2003; Zhang et al., 2004). Organelle structures, particularly mitochondria, are mostly preserved because apoptosis is an energy-consuming process (Friberg & Wieloch, 2002).

Coded proteins that are inactivated by covalent modifications or interactions with other anti-apoptotic regulatory molecules are necessary for pro-apoptotic signalling. Cell death stimuli are able to bring about cellular changes that remove the covalent modifications and block binding of anti-apoptotic regulators, thereby effecting apoptosis. In neurons,

apoptosis can be carried out through a variety of discrete pathways, which can be categorized as either intrinsic or extrinsic. In neurons, intrinsic pathways can be triggered by intracellular damage that is caused by free radicals or excitotoxicity; extrinsic death pathways can be activated by tumour necrosis factors (TNF) or lack of neurotrophins and other growth factors. Once activated, both intrinsic and extrinsic pathways can trigger caspase-dependent or caspase-independent cell death.

3.3.1 Apoptotic pathways

The caspase family of proteases is the most common and best understood mediators of apoptosis. In humans, at least seven caspases are implicated in apoptosis, including the initiator caspases 2, 8, 9, and 10, and the executioner caspases 3, 6, and 7 (Kroemer & Martin, 2005). Activated initiator caspases are able to cleave themselves and downstream targets, causing a cascade of caspase activation culminating at the executioner caspases, which have cell structures as their substrates and directly induce apoptosis. Caspase-activated deoxyribonuclease (CAD) causes the characteristic laddered DNA fragmentation observed when its inhibitor, ICAD, is cleaved by executioner caspases (Liu et al., 1997; Liu et al., 1999).

Following cerebral ischemia, the caspase cascade can be initiated early on through cell-death receptors or by mitochondrially mediated pathways (Ashkenazi & Dixit, 1998). The two mechanisms are not necessarily mutually exclusive and can be activated sequentially depending upon cell type and insult stimuli.

The Fas receptor, a primary death receptor in ischemia-induced apoptosis (Ferrer & Planas, 2003), belongs to the TNF receptor (TNFR) family and is specific for the Fas ligand (FasL) expressed on T cells. Activation of the Fas receptor causes formation of the cell-death-inducing signalling complex (DISC), which activates caspase-8 through the Fas-associated death domain (FADD). Caspase-8 can then activate caspase-3 to bring about apoptosis or activate the mitochondrial death pathway by cleaving Bid, a promoter for mitochondrial apoptosis-induced channel (MAC) formation (Planas et al., 1997). TNFR1 is also a member of the TNFR family, which induces apoptosis through a similar mechanism (Stanger et al., 1995). The upstream activators of the TNFRs in stroke models are increased during inflammation and include FasL and TNF-α.

The mitochondrial pathway is activated by inducing MOMP through the formation of the MAC, which is thought to be an oligomerized product of the Bcl-2 proteins Bax and Bak (Dejean et al., 2010; Martinez-Caballero et al., 2009). Regulation of pore formation is carried out by the Bcl-2 family, which includes anti-apoptotic proteins Bcl-2 and Bcl-xL and pro-apoptotic proteins Bid (which is cleaved to become the active tBid), Bim, and Bad (Gross et al., 1999; Imazu et al., 1999; Susin et al., 1996; Yang et al., 1997). Upon formation, the MAC allows cytochrome *c* release to the cytoplasm, where it interacts with Apaf-1 and dATP to form apoptosomes that cleave and activate caspase-9 (Zou et al., 1997). Caspase-9 then activates executioner caspases 3, 6, and 7 to bring about apoptosis. Smac/DIABLO and HtrA2/Omi are also released from the mitochondria along with cytochrome *c* (Du et al., 2000; Suzuki et al., 2001; Verhagen et al., 2000). Both promote apoptosis by respectively removing inhibitor of apoptosis protein (IAP)'s and X-linked inhibitor of apoptosis protein (XIAP)'s inhibition of caspase-3 and caspase-9 (Suzuki et al., 2001). The action of Smac/DIABLO is inhibited by Bcl-2 and Bcl-xL, which gives some degree of control over apoptosis even after the activation of the MAC.

The mitochondrial permeability transition pore (mPTP) is activated by excess Ca^{2+} levels, loss of voltage between inner and outer mitochondrial membranes, and high levels of free radicals. It is regulated by Bcl-2 proteins and is capable of releasing cytochrome *c* to bring about caspase-dependent apoptosis. The mPTP is often associated with excitotoxicity, which provides the requisite levels of Ca^{2+} needed to induce the mPTP to open (Ichas & Mazat, 1998; Martin, 2011). The mPTP is associated with cytochrome *c* release and various reactive oxygen species (ROS). It is involved in oxidative-stress-mediated apoptosis (Baumgartner et al., 2009).

3.4 Necrosis-like cell death

Despite the prevalence of apoptosis in delayed neuronal death, there is another cell-death pathway capable of inducing cell death independently of caspase activation (Kim et al., 2005a; Kroemer & Martin, 2005; Lang-Rollin et al., 2003; Le et al., 2002; Lockshin & Zakeri, 2002). Because it is with features of necrosis, the caspase-independent cell death is also known as necrosis-like cell death (Vande Velde, et al. 2000). Preventing caspase activation by using broad caspase inhibitors such as zVAD-fmk or testing with caspase 3 or 9 knockouts provides only minor protection against cell death after brain ischemia (Himi et al., 1998; Kim et al., 2005b; Le et al., 2002). Dying neurons in the penumbra exhibit 50 kbp DNA fragments, which is atypical of the caspase-dependent chromatin fragmentation that usually results in fragments of 200-1000 bp (MacManus et al., 1997). These findings indicate that caspase-independent, or necrosis-like, cell-death pathways are probably involved in delayed neuronal death. AIF and EndoG may be important players in necrosis-like cell-death pathways (Cande et al., 2002; van Loo et al., 2001).

AIF is a mitochondrial protein localized in the inner mitochondrial membrane, where it is an oxidoreductase. Upon mitochondrial permeabilization, AIF is released into the cytoplasm and subsequently translocates into the nucleus, where it contributes to chromatin condensation and fragmentation (Krantic et al., 2007). The fragments produced are 50kbp in size, which is consistent with observations of caspase-independent cell death (Cao et al., 2003). Activation of the cell-death pathway ending in AIF is also independent of caspases, since the broad inhibitors zVAD-fmk and zDEVD-fmk do not provide neuroprotection. Inhibition of AIF or knockdown of AIF expression is able to protect against stroke-like conditions (Culmsee et al., 2005). Since AIF relies on passage through the MAC pore to cause cell death, the same regulators of the caspase-dependent mitochondrial pathway are applicable (Tsujimoto, 2003). Bcl-xL prevents AIF translocation to the nucleus (Cao et al., 2003), while tBid and Bax cause AIF efflux from the mitochondria (Cregan et al., 2002; van Loo et al., 2002). Since AIF is attached to the inner mitochondrial membrane, AIF is cleaved from the membrane before it can leave through mitochondrial pores (Donovan & Cotter, 2004). This step is not well-understood, though it is known to be caspase-independent and possibly carried out by tBid and Bax (Donovan & Cotter, 2004; Otera et al., 2005).

Endonuclease G (EndoG) is another well-established mediator of caspase-independent cell death (Li et al., 1997; van Loo et al., 2001). EndoG acts after transient cerebral ischemia (Lee et al., 2005) and oxygen-glucose deprivation (Tanaka et al., 2005), while working independently of caspase-activated DNase (Li et al., 2001; van Loo et al., 2001). Like AIF, EndoG is present in the mitochondrial inter-membrane space, localizes to the nucleus upon release, and causes cell death by cleaving chromatin into fragments. The Bcl-2 family moderates EndoG release from the mitochondria (Donovan & Cotter, 2004); tBid can cause

EndoG efflux. The pro-apoptotic protein BNIP3 causes caspase-independent cell death in hypoxia and stroke through the action of EndoG (Zhang et al., 2007b).

3.4.1 The BNIP3-activated and EndoG and AIF-mediated neuronal death pathway

BNIP3 is part of a unique subfamily of death-inducing mitochondrial proteins that includes BNIP3, NIX, BNIP3h and a Caenorhabditis elegans ortholog, ceBNIP3. Expression of BNIP3 can induce death of various cells (Chen et al., 1997), including neurons (Zhang et al., 2007a; Zhang et al., 2007b; Zhang et al., 2007c). Cell death mediated by BNIP3 is characterized through cell transfection studies by early permeabilization of the plasma membrane and damage to the mitochondria without release of cytochrome *c* or activation of caspases (Cizeau et al., 2000; Ray et al., 2000). BNIP3 triggers mPTP opening, decreases mitochondrial membrane potential, and increases generation of ROS once it localizes to the mitochondrial outer membrane (Vande Velde et al., 2000).

The BNIP3 protein has four domains: a PEST domain that targets BNIP3 for degradation, a putative Bcl-2 homology 3 (BH3) domain that is homologous to those on other members of the Bcl-2 family, a CD domain that is conserved from *C. elegans* to humans, and a C-terminal transmembrane domain that is necessary for its mitochondrial localization and for its cell-death-inducing activity (Chen et al., 1999; Farooq et al., 2001; Yasuda et al., 1998). The BH3 domain is often necessary for Bcl-2 proteins to mediate cell death. BNIP3 possesses a BH3 domain that is not necessary for its cell-death-inducing ability in vivo and in vitro (Cizeau et al., 2000; Ray et al., 2000). The mechanism may operate independently of interaction with the Bcl-2 family.

BNIP3 is not detectable in normal neurons but is inducible under hypoxia in a variety of cells and tissues (Bruick, 2000; Guo et al., 2001; Sowter et al., 2001). The BNIP3 promoter contains a functional HIF-1 response element (HRE) that is activated by either hypoxia or forced expression of HIF-1α (Bruick, 2000). HIF-1α accumulation and subsequent activation of BNIP3 is induced by oxidative stress (Zhang et al., 2007a).

HIF-1 is a basic helix-loop-helix PAS domain (BHLH-PAS) transcription factor that normally regulates homeostatic responses to hypoxia in cells (Greijer & van der Wall, 2004). HIF-1 is composed of HIF-1α and HIF-1β; HIF-1 requires heterodimerization of both to function. HIF-1β is constitutively expressed, while HIF-1α expression and stability is dependent on intracellular oxygen levels. Under hypoxia, HIF-1α stabilizes and binds to HIF-1β in order to form HIF-1, which activates genes with HREs in their promoters.

Our work shows that hypoxia increases both BNIP3 and HIF-1α levels in neurons and that knockdown of HIF-1α expression is able to protect cells from hypoxia-induced death (Z. Zhang et al., 2007). Delayed neuronal death is also reduced when cortical neuron cultures are given a dominant-negative form of HIF-1α (HIFdn) via a herpes amplicon (Halterman et al., 1999). Our proposed pathway and other major caspase-independent pathways are shown in figure 1.

4. Therapy

The root of ischemic damage can be traced to a loss of adequate oxygen and glucose due to interrupted blood flow. While this is a singular event responsible for most, if not all, subsequent neuronal death, it is unrealistic to design treatments that are able to restore blood flow in the few seconds to minutes before any damage occurs. Rather, treatment must focus on either prophylactic manipulations of these mechanisms or downstream pathways

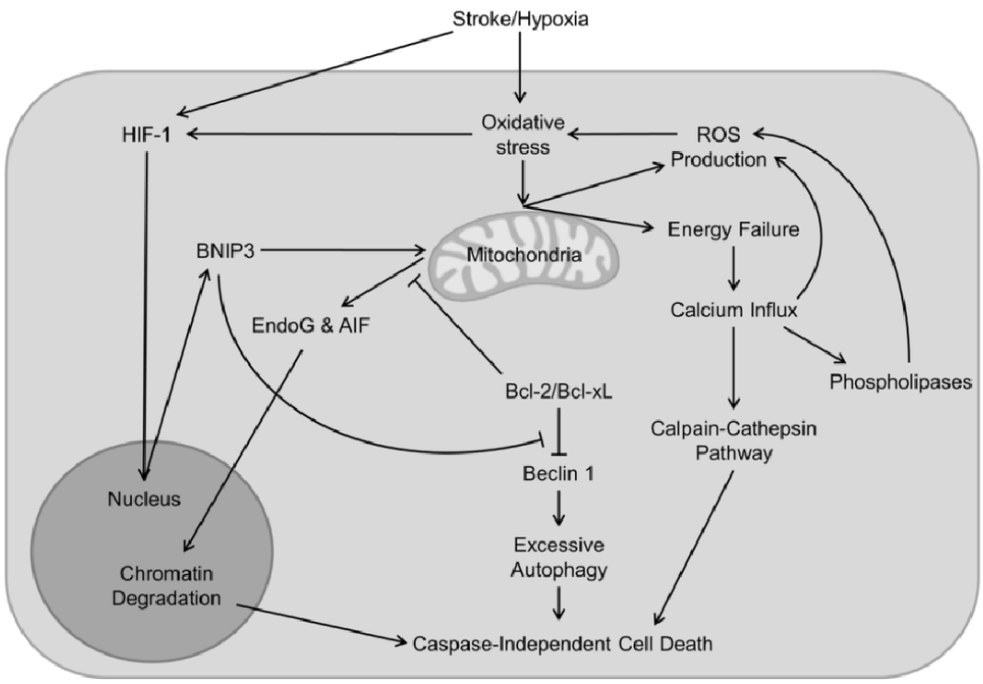

Fig. 1. Caspase-independent cell-death pathways. HIF-1 is induced directly by hypoxia or by oxidative stress and activates the expression of BNIP3 to cause the mitochondrial release of EndoG and AIF. Translocation of EndoG and AIF to the nucleus results in neuronal cell death without cytochrome c release and caspase activation. Bcl-2/Bcl-xL normally binds with beclin 1 to inhibit its activity. Sufficient BNIP3 displacement of Bcl-2/Bcl-xL from beclin 1 can cause runaway autophagy resulting in cell death. Immediate energy failure following stroke or hypoxia results in calcium disregulation and influx, triggering ROS production, phospholipase activity, and the calpain-cathepsin pathway. These processes can effect caspase-independent cell death.

that involve oxidative stress, energy depletion, ion deregulation, loss of protein synthesis, and activation of a host of protective and cell-death-inducing internal cell mechanisms. Due to the complex interactions that lead to delayed neuronal death in stroke, multi-approach strategies must be used. A comprehensive approach targeting as many pathways as possible would theoretically yield the best patient outcomes.

4.1 Antioxidants

Targeting a wide range of proteins and mechanisms involved in oxidative stress may provide beneficial therapeutic interventions for ischemia and reperfusion injury. Application of antioxidant compounds appears to be effective in combating oxidative stress in stroke (Huang et al., 2001). Antioxidant enzymes may protect against apoptosis after cerebral ischemia and reperfusion. Superoxide dismutase (SOD) has a protective role against focal cerebral ischemia. SOD-1 overexpression attenuates apoptotic cell death (Saito et al., 2004).

Melatonin is known for its neuroprotective free radical scavenging and antioxidant properties and may be a candidate for protecting against delayed neuronal death. Melatonin can readily cross the blood–brain barrier and effectively prevents neuronal loss in

experimental models of ischemia and in various in vivo focal and global ischemia/reperfusion models (Letechipia-Vallejo et al., 2001; Sinha et al., 2001). Melatonin's protective mechanism may lie in its ability to bolster intracellular antioxidative mechanisms. Glutathione peroxidase activity is upregulated by melatonin, as are the gene expressions of Mn-SOD and Cu/Zn-SOD, while preventing the activation of the transcription factor NF-κB. Oxidative stress can activate several cell signalling cascades that may trigger further damage and cell-death programs. Targeting the messengers that mediate this crosstalk may prove as a viable strategy for preventing cell death. The mitogen-activated protein kinases (MAPKs) such as p38, ERK, and JNK/SAPK are important mediators of cell survival and death following ischemic injury; their activation can lead to cell death. Inhibition of their activity reduces cell damage and results in neuroprotection. Other immediate events downstream of oxidative stress, such as degradation of membranes and production of arachidonic acid by phospholipases, may be potential therapeutic targets in stroke.

4.2 Autophagy-related therapy

Blocking autophagy more than four hours after cerebral ischemia can be neuroprotective (Puyal & Clarke, 2009), despite controversy about autophagy's role as a protective or damaging mechanism. 3-methyladenine, injected intracerebroventricularly following stroke, reduces the volume of the lesion by almost half, even when given hours after a stroke has occurred. Knockdown of Atg7, the gene coding for beclin 1, also provides protection against hypoxia and ischemia (Koike et al., 2008; Nitatori et al., 1995). Other methods of downregulating beclin 1, and even BNIP3, should yield protection against autophagic cell death. Since there is the possibility that autophagy serves a mainly protective function in some neurons, too broad or unspecific an inhibition may exacerbate injury from stroke. More research needs to be done to determine the exact effects of blocking these autophagy inducers.

4.3 Hsp-related therapy

Hsp70 expression is related to a neuron's ability to survive an ischemic insult. During ischemia, Hsp expression depends on activation of NMDA receptors (Ahn et al., 2008; Lipton, 1999; Saleh et al., 2009). While this receptor is an attractive target for neuroprotection, it must be noted that NMDA receptor overstimulation may play a major role in excitotoxicity, since it mediates calcium influx. If treatment strategies are to be pursued, a balance must be established between the activation of Hsp70 expression and exacerbation of excitotoxic damage. A possible solution is to use melatonin, which is capable of inducing Hsp70 upregulation and has antioxidative effects. Gene therapy to induce the expression of Hsp72 is effective in mice and may also be an option once the technology becomes more mature.

Hsp-related therapy primarily relies on preconditioning. Hsps have protective effects only when they exist at sufficient levels in the cytoplasm. That is an unlikely scenario for a patient during a stroke, where no precondition has occurred, ischemic onset is quick and severe, and protein synthesis is halted or slowed. Most of the evidence for Hsp neuroprotection involves pretreatment to induce Hsp expression prior to the ischemic insult. Hsp-based treatment might find utility during reperfusion, if its expression can be induced rapidly and sufficiently and is shown to offer protection against this second wave of injury.

A recent study has implicated Hsp70 in blocking the release of AIF from the mitochondria. This may be an additional mechanism for preventing delayed neuronal death by inhibiting the activation of caspase-dependent cell-death pathways (Ruchalski et al., 2006).

4.4 Protective effects of exogenous growth factors

Neurotrophins, like Hsps, are exploitable as neuroprotective elements. Exogenous BDNF protects against delayed neuronal death in the rat (Beck et al., 1994; Tsukahara et al., 1994) after ischemia. Administration of VEGF is neuroprotective through inhibition of apoptosis (Hayashi et al., 1998; Jin et al., 2001; Manoonkitiwongsa et al., 2004). Gene therapy strategies for GDNF are also promising (Harvey et al., 2005; Shirakura et al., 2003; Tsai et al., 2000). Other neurotrophins are similarly able to exert protective actions by inhibiting death or triggering protective mechanisms. Neurotrophins suffer the drawback of being difficult to deliver. Many require administration before or immediately after an ischemic incident to be effective. Various methods have been devised to target neurotrophins to neurons in order to reduce delayed neuronal death.

Neurotrophins are difficult to localize to the neurons in a clinical setting. Most do not cross the blood-brain barrier, and large doses to overcome the minimal localization to brain neurons result in harmful side effects (Ferrer, 2006). The use of viral or ligand vectors to carry neurotrophins have had some success in ischemic models. Murine monoclonal antibody against rat transferrin receptors (OX26-SA) linked to a neurotrophin is capable of neuroprotection when injected into the carotid arteries, though treatment must be promptly administered after ischemia to observe any protective effects (Wu, 2005). Targeting also allows for lower doses to be used, which overcomes the obstacle of otherwise inducing side effects.

At least one study has found an increase in neuronal necrosis following BDNF pre-treatment in cell culture while reducing apoptosis in the same cells (Koh et al., 1995). The mechanism may be via the potentiation of NMDA-mediated Ca^{2+} influx, which can amplify excitotoxic effects. Another explanation may be that BDNF exacerbates free-radical-induced cell death (Gwag et al., 1995). A patient who has suffered a stroke would virtually never have received pre-treatment with neurotrophins, but the fact that neurotrophins could inadvertently exacerbate damage under certain conditions (Gwag et al., 1995) should be considered when designing neuroprotective strategies.

4.5 Caspase inhibitors

Caspases and their associated players in apoptosis may also be viable targets for preventing delayed neuronal death. Caspase inhibitors, such as the specific caspase-1 inhibitor Ac-WEHD-CHO, and broad capase inhibitors, such as z-VAD-fmk, protect against delayed neuronal death in CA-1 pyramidal cells (Hayashi et al., 2001). Injection of benzyloxycarbonyl-Asp-CH2-dichlorobenzene, a permanent inhibitor of caspases, also offers protection against delayed neuronal death by delaying chromatin condensation and DNA fragmentation (Himi et al., 1998). Administration of the broad inhibitors z-VAD-fmk and z-DEVD-fmk preserves neurological functions in addition to attenuating delayed death (Endres et al., 1998). Upregulation of the activity of intracellular caspase inhibitors is also an option. Induced overexpression of XIAP using viral vectors shows neuroprotective effects (Xu et al., 1999). UCF-101, an HtrA2/Omi inhibitor, prevents apoptosis by regulating Fas-mediated proteins in extrinsic apoptosis as well.

These caspase inhibitors can be a valuable tool to combat delayed neuronal death, despite many of them being unable to cross the blood brain barrier. Most studies involve the direct injection of the inhibitors into brain tissue or intraventricular space. Seeing as intrinsic caspase-dependent cell death depends on mitochondrial permeability, there is a chance that blocking caspase activation may allow caspase-independent death pathways to occur. As a result, inhibiting only caspases may allow a number of cells to die by alternative means. Caspase inhibitors alone do not help in preserving long-term potentiation and plasticity of neurons after ischemia (Gillardon et al., 1999). Theoretically, blocking as many of the cell death signalling pathways as possible may maximize neuroprotection.

A concern with the use of caspase inhibitors in therapy or inhibiting apoptosis in general is that it may increase the probability of developing cancer or autoimmune disorders. This risk must be balanced against the potential neuroprotective effects of directly inhibiting apoptosis. This risk may be minimized if the inhibitors are localized as much as possible to the infarct region.

4.6 AIF and EndoG

Recently, more therapeutic strategies have been targeted towards caspase-independent cell death that is mediated by AIF and EndoG. Reducing the levels of AIF in a cell by using neutralizing antibodies (Cregan et al., 2002), RNAi (Strosznajder & Gajkowska, 2006) or gene knockout (Klein et al., 2002) is strongly neuroprotective. Downregulation of EndoG activity has been explored. Our team has found that RNAi inhibition of BNIP3 reduces EndoG translocation and is neuroprotective against hypoxia-induced cell death (Zhang et al., 2007b). Other studies have found that mutant heterozygosity for EndoG in transgenic mice provides resistance to TNF-α-induced cell death (Zhang et al., 2003).

AIF and EndoG release can be inhibited by preventing mitochondrial outer membrane permeabilization (MOMP). Blocking MAC activation or preventing mitochondrial rupturing may be neuroprotective. Seeing as most stroke patients are treated for hours after a stroke occurs, when MOMP has already been induced, strategies centred on preventing mitochondrial release of death promoters are limited. Some benefit may still exist for those cases receiving prompt intervention, when treatment can prevent MOMP in affected but not yet compromised mitochondria. Preventing MOMP while simultaneously targeting downstream death effectors may prevent cell death (Galluzzi et al., 2009).

Hsp70 is capable of inhibiting AIF release from the mitochondria. This mechanism may be dependent on the C-terminal region of Hsp70 rather than its enzymatic activity (Sun et al., 2006). Hsp70 may be capable of inhibiting the nuclease functions of EndoG in an ATP-dependent manner as well (Kalinowska et al., 2005). Hsp70 may offer neuroprotection through a multitude of pathways.

MOMP inhibition by targeting upstream factors has achieved significant levels of neuroprotection in vivo and is another therapeutic possibility. For example, it has been found that inhibiting the family of MAPKs can protect against ischemic damage. Treating mice through inhibition of p53 by genetic (Morrison et al., 1996), pharmacological (Culmsee et al., 2001) means, or by using blockers of the JNK signalling pathway (Gao et al., 2005; Guan et al., 2006) has resulted in neuroprotection against ischemia and excitotoxicity, presumably in part by reducing mitochondrial permeability.

4.7 MOMP prevention by targeting Bcl-2 proteins

Regulating Bcl-2 proteins provides protection against delayed neuronal death by preserving mitochondrial integrity. The MAC pore is key in regulating mitochondrial permeability and is under the control of the Bcl-2 family of proteins. Inhibition or upregulation of select members by genetic or pharmacological means can modulate the downstream activation of caspase-dependent apoptosis and AIF- or EndoG-mediated necrosis-like cell death; and they have been investigated for treatment strategies.

Inhibiting the pro-apoptotic BH3-only Bcl-2 proteins prevents MOMP, providing protection against mitochondria-mediated cell death. Pharmacologically blocking Bid with 4-phenylsulfanyl-phenylamine derivatives prevents tBid-induced Smac release, AIF release, caspase-3 activation, and nuclear condensation (Culmsee & Plesnila, 2006; Culmsee et al., 2005). Knockouts of Bid (Plesnila et al., 2002; Plesnila et al., 2001) or Bax genes (Gibson et al., 2001; Tehranian et al., 2008) protect against ischemic cell death in stroke models as well.

Genetic means of boosting the effects of Bcl-2 antiapoptotic proteins provide neuroprotection. Transgenically upregulating the protective Bcl-2 gene provides protection in mice of neurons injured by ischemia (Martinou et al., 1994). A similar effect can be observed when human Bcl-2 is overexpressed with herpes simplex virus vectors (Linnik et al., 1995). Gene therapy using adeno-associated viruses carrying the Bcl-2 gene is also effective (Shimazaki et al., 2000). Human gene therapy, while powerful, is not yet mature, so it will take time for these approaches to be proven effective and integrated into a clinical setting. In the meantime, other methods of upregulating protective Bcl-2 members should be explored.

BDNF is capable of regulating cell-death pathways. BDNF is capable of counter-regulating Bax and Bcl-2 when administered intravenously after ischemia (Schabitz et al., 2000). Neuroprotection is achieved by conjugating the product of the bcl-x gene with the HIV-Tat PTD as a method of delivery (Asoh et al., 2002). The upregulation of Bcl-2 and downregulation of Bax is implicated as part of hypothermia's protective mechanism against ischemic damage. These and other methods for regulating the Bcl-2 family may prove clinically relevant and could be examined for extra neuroprotection when used in combination or with therapies targeting different cell death mechanisms.

4.8 Excitotoxicity and calcium-mediated damage prevention

Excitotoxic damage due to massive Ca^{2+} influx should be reduced to some extent by either preventing ion disturbance or targeting the resulting structural damage by calpains, free radical production, and caspase activation. Free radical production and caspase activation may demonstrate protective action for mild excitotoxic stress causing delayed neuronal death.

Neuroprotection by blocking glutamate release and reception has been attempted with some success. In experimental stroke models, glutamate blockade provides protection against cell death, but the results do not necessarily translate into human therapy. Blocking the release of glutamate and other excitatory amino acids through the use of various drugs has been unsuccessful in human trials. Too little is known about neuroprotection to rule out glutamate blockers entirely. It is possible that treatment in these trials occurred too late and was unable to block the excitotoxic chain reaction. If the drugs were administered within a couple hours of stroke, then the time conditions would be similar to those found in models

used in studies successfully demonstrating neuroprotection (Babu & Ramanathan, 2011; Prass & Dirnagl, 1998).

Some potential therapeutic targets may alleviate calcium-induced neuronal damage. Calcium/calmodulin-dependent protein kinase kinase (CaM-KK) protects against delayed apoptosis following glutamate by activating Atk and CaM kinase IV (Yano et al., 2005), which both are anti-apoptotic players. Nimodipine (Mossakowski & Gadamski, 1990; Nuglisch et al., 1990), dantrolene (Wei & Perry, 1996), and the tetrapeptide Tyr-Val-Ala-Asp-chloromethyl ketone (Ac-YVAD-cmk) (Gray et al., 2001) are all able to block damage due to high cytosolic Ca^{2+} levels in a variety of stroke models and may be useful in preventing excitotoxic damage.

Energy depletion plays a large role in excitotoxicity. Methods that selectively inhibit poly (ADP-ribose) polymerase-1 (PARP-1) and PARP-2 offer neuroprotection (Chiarugi, 2005) by counteracting energy-consuming activities following ischemia and reducing the drop in high-energy molecules. Additional evidence implicates PARP in a pathway capable of inducing AIF release and activation (Niimura et al., 2006), which indicates that therapies targeting PARP may have a protective effect against AIF-mediated delayed neuronal death. Drugs that inhibit PHRP activation, such as PJ34 (Xu et al., 2010) or hepatocyte growth factor (Niimura et al., 2006), may be useful as part of multimodal early interventions.

5. Conclusions

Neuronal cell death following stroke occurs in necrosis, apoptosis and other alternative modes and is mediated through diverse molecular pathways. These pathways provide therapeutic targets for stroke management.

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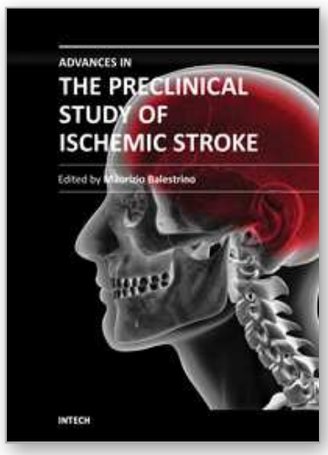

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This book reports innovations in the preclinical study of stroke, including - novel tools and findings in animal models of stroke, - novel biochemical mechanisms through which ischemic damage may be both generated and limited, - novel pathways to neuroprotection. Although hypothermia has been so far the sole "neuroprotection" treatment that has survived the translation from preclinical to clinical studies, progress in both preclinical studies and in the design of clinical trials will hopefully provide more and better treatments for ischemic stroke. This book aims at providing the preclinical scientist with innovative knowledge and tools to investigate novel mechanisms of, and treatments for, ischemic brain damage.

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