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Apoptotic Mechanisms After Cerebral Ischemia

Brad R.S. Broughton, PhD; David C. Reutens, MD; Christopher G. Sobey, PhD

Background and Purpose—Traditionally, cell death after cerebral ischemia was considered to be exclusively necrotic in nature, but research over the past decade has revealed that after a stroke, many neurons in the ischemic penumbra will undergo apoptosis.

Summary of Review—This brief review provides a general overview and update of various signaling pathways in the development of apoptosis in ischemic lesions. Cerebral ischemia triggers two general pathways of apoptosis: the intrinsic pathway, originating from mitochondrial release of cytochrome *c* and associated stimulation of caspase-3; and the extrinsic pathway, originating from the activation of cell surface death receptors, resulting in the stimulation of caspase-8. Although many of the key apoptotic proteins have been identified, our understanding of the complex underlying mechanisms remains poor and hence treatment of stroke patients by manipulating apoptotic pathways remains a daunting task. However, recent advances in the field have helped broaden our knowledge of apoptosis after cerebral ischemia. Further to the simplistic concept that stroke-induced apoptosis occurs predominantly in neurons and is caspase-dependent, accumulating evidence now indicates that apoptosis is prevalent in nonneuronal cells and that caspase-independent mechanisms also play a key role.

Conclusions—Although the ischemic penumbra is under threat of infarction, it is potentially salvageable and thus represents an opportunity for therapeutic intervention. (*Stroke*. 2009;40:e331-e339.)

Key Words: stroke ■ penumbra ■ caspases ■ cytochrome *c* ■ Fas receptor

Within minutes of a focal ischemic stroke occurring, the core of brain tissue exposed to the most dramatic blood flow reduction is fatally injured and subsequently undergoes necrotic cell death. This necrotic core is surrounded by a zone of less severely affected tissue which is rendered functionally silent by reduced blood flow but remains metabolically active.¹ This border region—known as the “ischemic penumbra”—may comprise as much as half the total lesion volume during the initial stages of ischemia, and represents the region in which there is opportunity for salvage via poststroke therapy.¹ Recent research has revealed that many neurons in the ischemic penumbra or periinfarct zone may undergo apoptosis after several hours or days, and thus they are potentially recoverable for some time after the onset of stroke. In contrast to necrosis, apoptosis appears to be a relatively orderly process of energy-dependent programmed cell death to dispose of redundant cells.² Cells undergoing apoptosis are dismantled from within in an organized way that minimizes damage and disruption to neighboring cells.

There are two general pathways for activation of apoptosis: the intrinsic and extrinsic pathways (see Figure 1). Over the last decade, experimental studies have provided considerable new information characterizing apoptotic processes occurring after ischemic stroke, and an overview of this will be described in this brief review. Differences in responses to

ischemia between mature and developing brain have also been described, but this review will focus on experimental findings from studies in mature animals. As targeting and preventing apoptosis in the penumbra would seem to be a rational therapeutic goal for limiting cerebral infarct volume after clinical stroke, the following recent information could provide a basis for design of novel interventions.

Intrinsic Mechanisms of Apoptosis

Channels Mediating Postischemic Ca²⁺ Entry

The onset of stroke restricts the delivery of substrates, particularly oxygen and glucose, and impairs the energetics required to maintain neuronal ionic gradients.³ Rapid depletion of energy after cerebral ischemia leads to a loss of membrane potential and depolarization of nerves. Voltage-dependent Ca²⁺ channels are then activated, and excitatory amino acids are released into the extracellular space.⁴ A cytotoxic accumulation of intracellular Ca²⁺ subsequently occurs and is thought to initiate a series of cytoplasmic and nuclear events, including the triggering of the intrinsic apoptotic pathway (Figure 2).³

Arguably, the most-studied ion channels in ischemic stroke are the group of receptor operated cation channels opened by glutamate, which accumulates in the extracellular space

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From the Department of Pharmacology (B.R.S.B., C.G.S.), Monash University, Victoria, Australia; and the Southern Clinical School (D.C.R.), Monash University, Clayton, Australia.

Correspondence to Christopher G. Sobey, PhD, Department of Pharmacology, Monash University, Wellington Road, Clayton, Victoria 3800, Australia. E-mail chris.sobey@med.monash.edu.au

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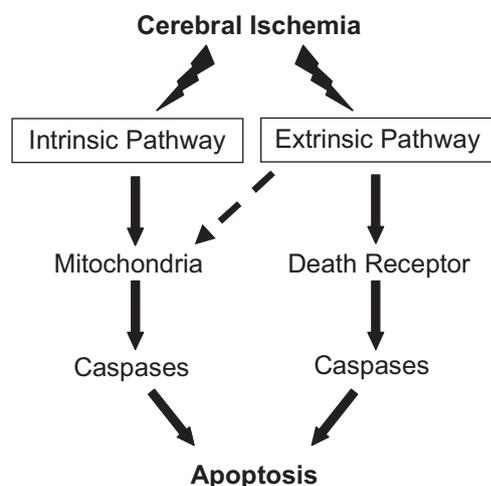


Figure 1. Apoptotic signaling cascades after cerebral ischemia. Apoptosis can be initiated by internal events (ie, “Intrinsic Pathway”) involving the disruption of mitochondria and the release of the cytochrome C, which leads to the downstream activation of caspases. Alternatively, cell surface receptors can be activated by specific ligands that bind to “death receptors” (ie, “Extrinsic Pathway”).

during cerebral ischemia.^{4,5} Binding of glutamate to ionotropic NMDA (*N*-methyl-D-aspartate) and AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors promotes a further increase in Ca^{2+} entry. Interestingly, although clinical trials with agents targeting glutamate receptors were disappointing,⁵ a possible explanation for those unfavorable clinical results was recently provided by Liu et al.⁶ Two types of NMDA receptors are present on neurons, NR2A and NR2B, and Liu and colleagues⁶ demonstrated that selective NR2B inhibition prevented NMDA-induced neuronal programmed cell death whereas NR2A receptor blockade failed to inhibit and in fact significantly increased NMDA-induced apoptosis. These surprising observations suggest that activation of NR2A-containing NMDA receptors may trigger cell survival-promoting mechanisms, counteracting the proapoptotic effect of NR2B receptor activation. Hence, the failure of NMDA receptor antagonist-based clinical trials for stroke may have been in part attributable to the “inadvertent” blockade of “prosurvival” NR2A-NMDA receptors.

The disappointing outcomes from clinical trials of NMDA receptor inhibitors and also L-type Ca^{2+} channel blockers⁷ led to interest in acid-sensing ion channels (ASICs) and transient receptor potential (TRP) channels as possible contributors to apoptosis of neurons after stroke. ASICs are ligand-gated cation channels permeable to Na^+ , and to a lesser degree Ca^{2+} , which respond to acidic stimuli. It seems likely that these channels play a role in apoptosis induced by cerebral ischemia, in which the degree of acidosis is severe.⁵ ASIC1a, the major ASIC subunit with Ca^{2+} permeability, and also ASIC2a, have been suggested to play a contributory role in cerebral ischemia.⁵ Intracerebroventricular administration of the ASIC1a inhibitors, amiloride and psalmotoxin 1, before induction of ischemic stroke, as well as knockout of the ASIC1a gene, is protective.⁸ At present, however, this line of evidence is only suggestive, and the specific targets of these drugs remain undetermined. Finally, evidence is emerg-

ing that TRP proteins may play a direct role in ischemic stroke, especially in Ca^{2+} -mediated neuronal death. More specifically, TRPM2 and TRPM7 may be important contributors to the delayed death of neurons after cerebral ischemia.⁵ Increasing evidence suggests that these channels potentially contribute to the activation of apoptotic proteins after cerebral ischemia via increased intracellular Ca^{2+} levels. Conversely, it was recently reported that TRPC3 and TRPC6 are involved in promoting neuronal survival.⁹ Whether TRPC channels play a neuroprotective role after cerebral ischemia remains to be delineated.

Effect of Increased Ca^{2+} on Mitochondria and the Release of Proapoptotic Proteins

The role of mitochondria in cerebral ischemic injury remains to be fully elucidated. However, several potentially deleterious mitochondrial responses have been identified after stroke.¹⁰ Examples include the inability to generate ATP¹¹ and the production of harmful radical species (see below). In addition, mitochondria are thought to be often centrally involved in the development of apoptosis. Mitochondria are considered to influence neuronal apoptosis primarily via the release of proapoptotic factors into the cytoplasm. Activation of calpains by increased Ca^{2+} or stimulation of caspase-8 via the extrinsic pathway (see below) results in the cleaving of Bcl-2 interacting domain (BID) to its truncated active form (tBID).^{12,13} BID is a cytosolic member of the Bcl-2 family of proapoptotic proteins that translocates to mitochondria when the cell receives a death signal.¹⁴ Recent studies have shown that BID is a critical mediator of ischemic cell death within neurons (Figure 2). For example, Plesnila and colleagues¹⁵ found evidence for BID cleavage in ischemic brain tissue and that deletion of the BID gene in mice reduced ischemic infarct size. tBID targets the outer mitochondrial membrane and induces conformational changes in other proapoptotic proteins, such as Bak, Bax, Bad, and Bcl-XS.¹⁴ These proapoptotic proteins can heterodimerise with tBID and antiapoptotic proteins, Bcl-2 or Bcl-XL, via their BH3 domains.¹⁶ In the brain shortly after the onset of ischemia, dephosphorylation and translocation of Bad from the cytosol to the mitochondria occurs and Bad dimerizes with Bcl-XL.¹⁷ Similar observations with Bax have been made in the ischemic brain.¹⁸ The mechanism(s) by which proapoptotic proteins induce the release of apoptogenic factors from the intermembrane space is undetermined, although it is believed to be through the opening of mitochondrial transition pores. Interestingly, it has been suggested that Bax can form channels in liposomes that are large enough to allow passage of cytochrome *c*.¹⁸

Caspase-Dependent Apoptosis

After the opening of the mitochondrial transition pores, two groups of normally sequestered proapoptotic proteins are released from the intermembrane space into the cytosol.¹⁹ The first group consists of cytochrome *c*, Smac/DIABLO, and the serine protease HtrA2/Omi, although we have little knowledge regarding these two latter proteins.¹⁹ Once released, these proteins activate the caspase-dependent mitochondrial pathway. Cytochrome *c* binds and activates the cytosolic protein Apaf-1 as well as procaspase-9, and together with

INTRINSIC APOPTOTIC PATHWAY

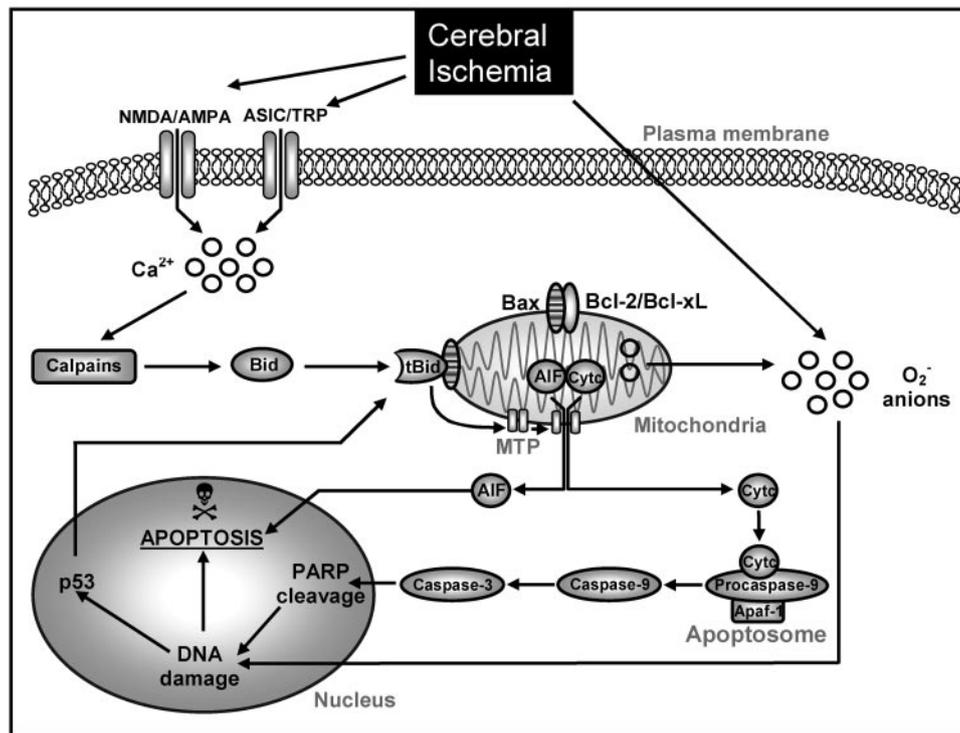


Figure 2. Intrinsic signaling cascade of apoptosis after cerebral ischemia. Cerebral ischemia elevates cytosolic calcium levels through the stimulation by glutamate of N-methyl-D-aspartate (NMDA) or D,L- α -amino-3-hydroxy-5-methyl-isoxazolpropionic acid (AMPA) receptors, or by activation of acid-sensing ion channels (ASICs). Increased intracellular calcium activates calpains and mediates cleavage of Bid to truncated Bid (tBid). At the mitochondrial membrane tBid interacts with apoptotic proteins such as Bax and Bcl-2, which is typically neutralized by antiapoptotic B-cell leukemia/lymphoma 2 (Bcl-2) family proteins Bcl-2 or Bcl-xL. After heterodimerization of proapoptotic proteins with tBid, mitochondrial transition pores (MTP) are opened, thus releasing cytochrome *c* (Cyto) or apoptosis-inducing factor (AIF). Once released into the cytosol, Cyto binds with apoptotic protein-activating factor-1 (Apaf-1) and procaspase-9 to form an “apoptosome,” which activates caspase-9 and subsequently caspase-3. Activated caspase-3 cleaves nDNA repair enzymes, such as poly (ADP-ribose) polymerase (PARP), which leads to nDNA damage and apoptosis. By contrast, AIF translocates rapidly to the nucleus where it mediates large-scale DNA fragmentation and cell death in a caspase-independent manner. In addition, nuclear pathways of neuronal apoptosis are activated in response to DNA damage, for example, through phosphorylation and activation of p53. Furthermore, cerebral ischemia and reperfusion generate superoxide anions (O_2^-), which causes DNA damage.

ATP they form an “apoptosome.”¹³ The clustering of procaspase-9 in this manner leads to caspase-9 activation. Caspase-9, which is presumably an initiator of the cytochrome *c*-dependent caspase cascade, then activates caspase-3.¹⁴

Considerable evidence suggests the release of mitochondrial cytochrome *c* to occur after induction of cerebral ischemia. For example, it has been reported that cytochrome *c* is translocated from the mitochondria to the cytosolic compartment after either transient or permanent focal cerebral ischemia in rats.²⁰ Furthermore, immunoprecipitation experiments revealed an enhanced formation of Apaf-1/caspase-9 complex in the hippocampus 8 to 24 hours after global ischemia.²¹ These investigators also demonstrated that the specific Apaf-1 inhibitor, Apaf-1 interacting protein, is neuroprotective in cerebral ischemia,²¹ and similar observations have also been reported after focal ischemia.²² Caspase-9 also plays an important role in neuronal death after cerebral ischemia, and Sugawara et al²³ demonstrated cleaved caspase-9 to be increased in brain 12 hours after ischemia.

Caspase-3 has been identified as a key mediator of apoptosis in animal models of ischemic stroke. Asahi and

coworkers²⁴ demonstrated upregulation of caspase-3 mRNA in rat brain 1 hour after the onset of focal ischemia. In addition, Namura and associates²⁵ detected caspase-3 and its cleavage products in mouse brain during early reperfusion after 2-hour middle cerebral artery (MCA) occlusion. Importantly, comparable observations have been extended to ischemic human brain tissue in that caspase-3 was upregulated after ischemia.²⁶ Both genetic disruption²⁷ and pharmacological inhibition of caspases^{28–30} have been found to have a strong neuroprotective effect in experimental stroke. Furthermore, Fink et al³¹ observed that caspase activation occurred up to 9 hours after brief MCA occlusion, and that the ischemic damage could be reduced by caspase inhibitors injected up to 9 hours after reperfusion. These findings suggest that an extended treatment window for caspase inhibition is plausible after stroke.

Caspase-3 cleaves many substrate proteins, including poly (ADP-ribose) polymerase (PARP).^{25,32} PARP inactivation after cleavage by caspase-3 leads to DNA injury and subsequently to apoptotic cell death (see below). On the other hand, excessive activation of PARP causes depletion of nicotinamide-adenine dinucleotide and ATP, which ulti-

mately leads to cellular energy failure and necrotic cell death.¹⁴ Thus, genetic deletion or pharmacological inactivation of PARP reduces cerebral infarct volume after MCA occlusion.^{32,33} Collectively, these findings provide a strong basis for concluding that cerebral ischemia activates the mitochondrial apoptotic pathway, characterized by changes in Bcl-2 family proteins, cytochrome *c* release, and caspase-like enzyme activation.

Caspase-Independent Apoptosis

In addition to cytochrome *c*/caspase-3-mediated apoptosis, increasing evidence implicates a significant role for caspase-independent pathways in programmed cell death. Such a mechanism includes a second group of proapoptotic proteins released via the mitochondrial transition pores, such as apoptosis inducing factor (AIF), endonuclease G and Bcl-2/adenovirus E1B 19 kDa-interacting protein (BNIP3).¹⁹ Of these proteins, AIF is probably the best understood. There is conclusive evidence that AIF-induced cell death processes are not dependent on functional caspases and could therefore serve as an alternative death pathway after cellular energy depletion that precludes caspase activation.³⁴ This is supported by findings from Daugas et al,³⁵ who demonstrated that depletion of ATP acts as a stimulus for AIF release. Once released from the mitochondria, AIF translocates to the nucleus and causes large-scale DNA fragmentation and peripheral condensation of peripheral nuclear chromatin, which is distinct from the global chromatin condensation and oligonucleosomal DNA fragmentation of caspase-dependent death.³⁴ These differences indicate that AIF causes a unique type of death via a novel intracellular pathway. This raises the question, however, as to whether it remains appropriate to still classify this process as "apoptotic." Coincidentally, some groups have termed it "programmed necrosis" whilst others continue to refer to it as apoptosis.³⁴

Caspase-independent neuronal apoptosis has been documented after ischemic stroke. For example, Culmsee and colleagues¹² showed evidence of rapid AIF translocation to the nuclei of injured neurons after cerebral ischemia. In addition, specific inhibition of PARP-1 attenuated the migration of AIF and protected neurons from death after cerebral ischemia. Furthermore, reduced AIF protein levels in siRNA-treated neurons, or in harlequin mutant mice which express low levels of AIF, markedly reduced neuronal cell death after ischemia.¹² Collectively, these data suggest that PARP-1 is an upstream mediator of AIF-induced caspase-independent cell death in ischemic stroke, but the links between PARP-1 activation and AIF release remain to be elucidated. Furthermore, endonuclease G nuclear translocation has been associated with apoptosis after ischemic stroke. Lee et al³⁶ demonstrated that endonuclease G becomes detectable in the nucleus 4 to 24 hours after MCA occlusion. Findings from the same study also revealed that AIF and endonuclease G migrate from the mitochondria to the nucleus at about the same time postischemia.³⁶ These observations indicate that endonuclease G may be involved in apoptosis postcerebral ischemia, but the specific nature of the relationship between endonuclease G and AIF has yet to be established. Lastly, BNIP3 (a proapoptotic member of the Bcl-2 family which

contributes to mitochondrial dysfunction and cell death) mediates apoptosis independent of both caspases and AIF in primary neuron cultures exposed to hypoxic conditions.³⁷ This suggests that BNIP3 represents a novel caspase-independent apoptotic pathway in stroke that is separate from AIF. Together, the above findings indicate the importance of AIF, endonuclease G, and BNIP3 in caspase-independent cell death after ischemic stroke.

Although the release of cytochrome *c* and caspase-independent proteins from the mitochondria are a common consequence of the intrinsic apoptotic signaling pathway, there are alternative mechanisms by which mitochondrial dysfunction is likely to contribute to neuronal cell death. Brain cells after cerebral ischemia probably contain damaged mitochondria that are unable to maintain the electrochemical gradient necessary for respiration and glucose oxidation.³⁸ Indeed a significant reduction in ATP levels in the brain after ischemic episodes is well established.¹⁰ Consequently, these cells may exacerbate cell death by increasing energy failure. Interestingly, because apoptosis is an energy-dependent process,³⁹ it is possible that intrinsic apoptotic mechanisms may be activated in cells containing energy deficient mitochondria, but there may not be enough energy available to activate further downstream caspases. Hence, treatments that prevent mitochondrial dysfunction, in addition to caspase inhibition, should be considered as a potential protective strategy after cerebral ischemia.

Reactive Oxygen Species

Under physiological conditions reactive oxygen species (ROS), which include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), are generated at low levels and play important roles in signaling and metabolic pathways.⁴⁰ Intracellular sources of ROS include xanthine oxidase, mitochondrial electron transport chain, arachidonic acid, and NADPH oxidases.⁴¹ Importantly, ROS levels are controlled by endogenous antioxidants such as superoxide dismutases (SOD), glutathione peroxidase, glutathione, and catalase.⁴²

Increased levels of ROS are a major cause of tissue injury after cerebral ischemia, in which there is an overproduction of ROS, inactivation of antioxidant enzymes, and consumption of antioxidants, such that natural defense mechanisms fail to protect neurons from oxidative damage.⁴⁰ ROS may cause tissue damage after stroke, either directly through participation in destruction of cellular proteins, lipids, and DNA, or indirectly by disrupting normal cellular signaling and gene regulation. Interaction of ROS with other tissue components produces a variety of other radicals. Of particular importance is the interaction of O_2^- with nitric oxide, which produces the highly toxic molecule, peroxynitrite. This oxidant causes further tissue damage and is thought to be an important trigger molecule for apoptosis after ischemic stroke.³

It is generally thought that mitochondria are the primary source of ROS involved in ischemia-induced apoptosis. Studies have shown that mitochondrial ROS is produced by a variety of stimuli, including hypoxia itself, excitotoxicity (glutamate), and Ca^{2+} overload after cerebral ischemia.⁴ In addition, it has been reported that O_2^- generation is greatest

after reperfusion,⁴³ and postischemic reperfusion may particularly cause overproduction of ROS in mitochondria.⁴⁴ Once generated, mitochondrial ROS influence the release of cytochrome *c* and other apoptotic proteins from the mitochondria into the neuronal cytosol, which leads to apoptosis and defective gene expression after stroke.^{18,23} Thus, increasing evidence suggests that oxidative stress and apoptosis are closely linked phenomena in the pathophysiology of ischemic stroke.

There are numerous reports that ROS derived from NADPH oxidase play an important role in various tissues after ischemia-reperfusion.⁴¹ Although NADPH oxidase is known to be a source of increased O₂⁻ production after cerebral ischemia,^{41,45} no studies have yet examined whether O₂⁻ derived from NADPH oxidase activates the apoptotic signaling cascade in brain tissue. Another source of increased ROS generation that does appear to be related to apoptosis after cerebral ischemia is arachidonic acid metabolism.⁴⁶ Yagami et al⁴⁶ demonstrated that phospholipase A₂ enzymes that release arachidonic acid, known as group IIA secretory phospholipase A₂ (sPLA₂-IIA), induce apoptosis of neurons after stroke. This suggests that sPLA₂-IIA plays a crucial role in apoptosis of neurons in the penumbra of rats after ischemic stroke. Overall, the pathway of ROS-induced apoptosis represents another potential target for poststroke therapy.

DNA Damage

DNA damage, if irreparable, may activate apoptotic signaling mechanisms. In response to DNA damage, the tumor-suppressor transcription factor, p53, stops the cell cycle and triggers programmed cell death by promoting proapoptotic protein expression, suppressing antiapoptotic protein regulation, and targeting BH3-only proteins, p53-upregulated modulator of apoptosis (PUMA) and NOXA.⁴⁷ PUMA can inhibit all the antiapoptotic proteins and activate the intrinsic death pathway by switching on proapoptotic proteins, such as Bax and Bak.¹⁶ PUMA is activated at the transcriptional level by p53 and is induced by all stimuli that activate p53, including DNA damage and oxidative stress.¹⁶ Similarly, studies have shown that NOXA can translocate to the mitochondria and interact with antiapoptotic Bcl-2 family members, resulting in the activation of caspases.⁴⁷ Furthermore, it has been reported that p53 activates transcription of the Fas receptor after DNA damage, which raises the surprising notion that nDNA damage signals can be transduced via cell surface receptors.⁴⁸ It is interesting to note, however, that early p53-induced apoptotic cells can be rescued from the apoptotic program if the stimulus is removed.⁴⁹ Research by Geske and coworkers⁴⁹ suggested that DNA repair is activated early in the p53-induced apoptotic process and may be able to reverse the cell death pathway.

p53 is a promising target for stroke therapy, as it is rapidly upregulated in ischemic brain tissue where it initiates programmed cell death through the transcription of Bax, PUMA, or NOXA, as well as the subsequent mitochondrial damage and activation of caspases.⁴⁷ For example, Endo and colleagues⁵⁰ reported transcription-independent proapoptotic activity of p53 in a rat model of transient global ischemia, in which cell death occurred in hippocampal CA1 neurons after

the mitochondrial translocation of p53 and its interaction with the antiapoptotic Bcl-xL. In addition, it has been identified that PUMA expression is upregulated in regions of the hippocampus known to exhibit significant levels of apoptosis after cerebral ischemia, suggesting that PUMA could be a key target for poststroke therapy.⁵¹ Interestingly, harmful DNA damage-dependent signaling events, including p53 activation, and mitochondrial translocation of PUMA and NOXA, were reduced during postischemic reperfusion in hypothermia-treated rat brains.⁵² These findings suggested that DNA damage-triggered prodeath signaling events may be an important mechanism underlying the neuroprotective effect of mild hypothermia against ischemic brain injury.⁵²

Extrinsic Mechanisms of Apoptosis

Fas and TNF Receptor

Extrinsic mechanisms of apoptosis involve the engagement of death receptors located on the plasma membrane and is hence also referred as the “death receptor pathway” (Figure 3). Cell surface death receptors belong to the tumor necrosis factor receptor (TNFR) superfamily, and include TNFR-1, Fas, and p75^{NTR}.⁴⁰ Forkhead1, a member of the forkhead family of transcription factors, stimulates expression of target genes, such as the Fas ligand (FasL), which are implicated in the extrinsic receptor pathway of caspase-3 activation.¹⁴ FasL initiates apoptosis by binding to the Fas receptor, triggering recruitment of the cytoplasmic adaptor protein Fas-associated death domain protein (FADD). FADD contains a “death effector domain” at the N terminus which binds to procaspase-8 by interacting with its death effector domain.¹³ This complex (FasL–Fas–FADD–procaspase-8) is referred as death-inducing signaling complex (DISC) and is assembled within seconds of Fas receptor engagement. The signal complex catalyzes the proteolytic cleavage and transactivation of procaspase-8 to generate caspase-8.¹³ Once activated, caspase-8 is released from the DISC complex into the cytoplasm and initiates downstream cleavage of caspase-3 by direct or mitochondrial-dependent mechanisms, thus paving the way for the execution phase of apoptosis involving PARP cleavage, as discussed above.

There is now considerable evidence that cerebral ischemia triggers the extrinsic apoptotic signaling cascade. Fas, FasL, and TNF-related apoptosis-inducing ligand expression are upregulated in the postischemic rat brain.^{53,54} Interestingly, this upregulation was observed within 12 hours after cerebral ischemia and peaked between 24 and 48 hours, which coincides with the time course of apoptotic death in neuronal cells. Most importantly, mice expressing dysfunctional FasL,^{53,54} and also TNF knockout mice,⁵³ have significantly reduced cerebral infarcts after MCA occlusion. Remarkably, hybrid mice lacking both cytokines had a 93% reduction in infarct volume 24 hours after stroke.⁵³ A similarly profound level of protection was observed in wild-type mice treated with neutralizing antibodies against both FasL and TNF.⁵³ These findings emphasize the contribution of death receptors in cell death after cerebral ischemia.

EXTRINSIC APOPTOTIC PATHWAY

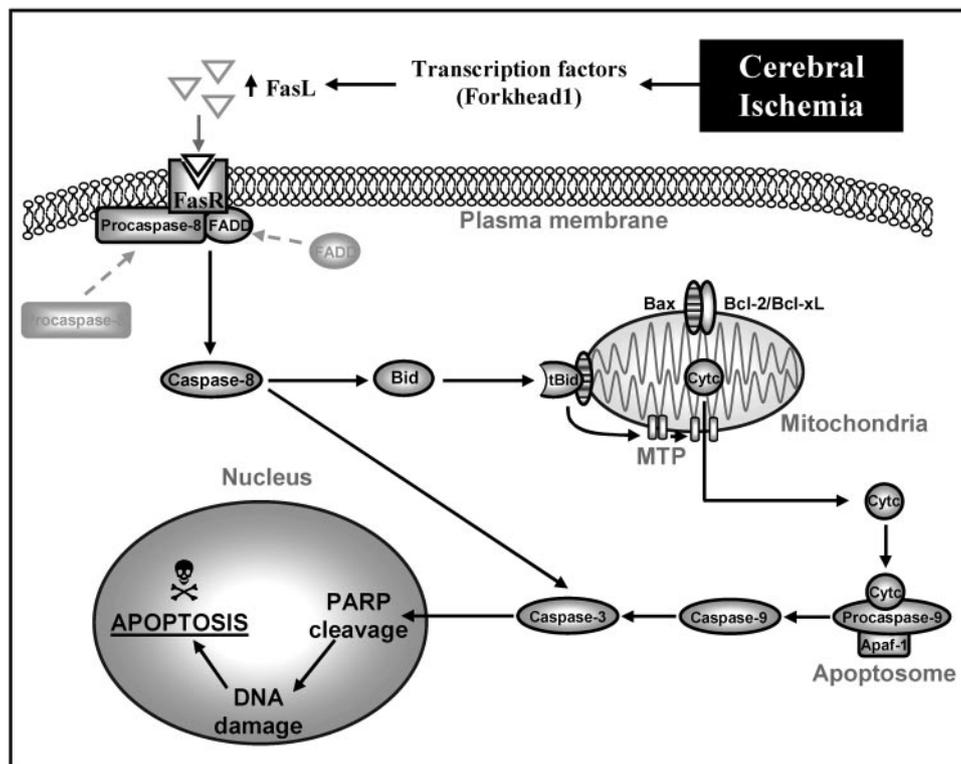


Figure 3. Extrinsic signaling cascade of apoptosis after cerebral ischemia. The extracellular Fas ligand (FasL) binds to Fas death receptors (FasR), which triggers the recruitment of the Fas-associated death domain protein (FADD). FADD binds to procaspase-8 to create a death-inducing signaling complex (DISC), which activates caspase-8. Activated caspase-8 either mediates cleavage of Bid to truncated Bid (tBid), which integrates the different death pathways at the mitochondrial checkpoint of apoptosis, or directly activates caspase-3. At the mitochondrial membrane tBid interacts with Bax, which is usually neutralized by antiapoptotic B-cell leukemia/lymphoma 2 (Bcl-2) family proteins Bcl-2 or Bcl-xL. Dimerization of tBid and Bax leads to the opening of mitochondrial transition pores (MTP), thereby releasing cytochrome c (Cyt c), which execute caspase 3-dependent cell death.

Apoptosis in Nonneuronal Cells

Our initial knowledge of apoptosis after cerebral ischemia was largely confined to its occurrence in neurons as early studies reported that almost all of the apoptotic cells in the ischemia core were identified as neuronal.^{55,56} However, there has been a steady increase in evidence relating to apoptosis in nonneuronal cells within the brain after stroke. For example, apoptosis of glial cells and infiltrating leukocytes after brain ischemia has been observed in some experimental models of stroke.¹³ Upregulation of caspase-3 and several other caspase family members has been reported in astrocytes and microglia after MCA occlusion.⁵⁷ Furthermore, Chen et al⁵⁸ identified DNA strand breakage in astrocytes in the border zone of the infarcted tissue after cerebral ischemia. However, the extent of ischemia-induced programmed cell death in glial cells remains uncertain and further research is required.

Similarly, little is known about programmed cell death in vascular tissue, although a small number of studies have examined apoptotic signaling mechanisms in vascular endothelial cells after ischemia-reperfusion. Diminished cerebral blood flow after cerebral ischemia triggers a series of processes that affects the microvasculature by disrupting the blood-brain barrier integrity and the regulation of arterial tone.³ As discussed above, large amounts of ROS, especially O_2^- , are generated in the brain after reperfusion, and ROS

have been reported to activate apoptosis in vascular endothelial cells.⁵⁹ In addition to the detrimental vascular actions of O_2^- in decreasing blood-brain barrier integrity and increasing vascular tone as mentioned above, its reaction with endothelium-derived nitric oxide to form the highly toxic peroxynitrite radical would be expected to trigger apoptosis in vascular cells after ischemic stroke.⁶⁰ Evidence for vascular apoptosis has also been reported after subarachnoid hemorrhage in endothelial cells of basilar arteries, in which double fluorescence labeling demonstrated colocalisation of TUNEL, which detects DNA fragmentation, with either caspase-3, caspase-8 or TNFR1.⁶¹ These observations suggest that the mechanism of apoptosis in cerebral endothelial cells after subarachnoid hemorrhage involves TNFR1 and the caspase-8 and caspase-3 pathways. There is now a need to characterize the features of apoptosis in cerebral vascular cells, and its consequences, after stroke. It is too early to predict whether specific or additional targeting of the vasculature to prevent apoptosis might represent a useful strategy for poststroke therapy.

Endogenous Antiapoptotic Mechanisms and Their Potential as Therapeutic Targets

Interactions between the proapoptotic and antiapoptotic Bcl-2 family proteins on the outer mitochondrial membrane are

believed to play an important role in cell survival.¹³ Typically, antiapoptotic members are located on the outer mitochondria membrane and suppress apoptosis through multiple mechanisms, including conserving mitochondrial membrane potential, inhibiting the release of apoptotic proteins, stabilizing the mitochondrial transition pores, thus preventing its opening, and controlling the activation of caspase proteases.¹⁶

Bcl-2 and Bcl-xL levels are downregulated within the first few hours of cerebral ischemia.¹⁶ Interestingly, significantly smaller infarcts have been observed in mice overexpressing Bcl-2 or Bcl-xL after focal cerebral ischemia,^{62,63} whereas stroke in Bcl-2 knockouts resulted in an increased infarct size.⁶⁴ Studies have demonstrated that various neuroprotective treatments reduce the impact of stroke via increasing expression levels of either Bcl-2 or Bcl-xL. For example, the administration of a Bcl-xL fusion protein that contains the TAT domain of the human immunodeficiency virus is protective in models of ischemia.⁶⁵ Furthermore, estradiol exerts profound protective effects on ischemic brain tissue and prevents the ischemic-induced downregulation of Bcl-2 expression, indicating that estradiol modulates the expression of Bcl-2 as a neuroprotective mechanism.⁶⁶ Collectively, these findings suggest the importance of antiapoptotic Bcl-2 family proteins in ischemic cerebral injury, and thus therapeutics targeting these proteins may potentially provide neuroprotection against stroke.⁴

It is important to note, however, that the neuroprotective effects of Bcl-2 and Bcl-xL can be neutralized through heterodimerization with proapoptotic proteins, such as Bad, which subsequently promotes cell death.¹⁴ Nevertheless, various upstream signaling mechanisms can stimulate transcription of antiapoptotic proteins or suppress the inhibitory effect of Bad on Bcl-xL. For example, phosphoinositide 3-kinase phosphorylates Akt, which subsequently phosphorylates Bad and eliminates its inhibitory effects on Bcl-xL in neurons after ischemia.¹⁴ Furthermore, extracellular signal-regulated kinase 1/2 (ERK1/2) inactivates Bad through phosphorylation of 90-kDa ribosomal S6 kinases.⁶⁷ Indeed, transforming growth factor- β 1 has been reported to phosphorylate and inhibit Bad activity via activation of the ERK pathway in brain during ischemia.⁶⁸ Consistent with those observations, Kilic and coworkers⁶⁹ using a transgenic mouse that expresses elevated levels of the human hematopoietic growth factor, erythropoietin, in the brain demonstrated that erythropoietin protects against focal cerebral ischemia via activating ERK1/2 and Akt signaling. In the same study, Bcl-xL expression in ischemic brain tissue was elevated above the levels in wild-type mice, suggesting that ERK1/2, Akt, and Bcl-xL pathways play a role in the neuroprotective function of erythropoietin.⁶⁹ Protein kinase A (PKA) can also phosphorylate Bad after cerebral ischemia, thus triggering its dissociation from Bcl-xL.¹⁷ The cumulative evidence presented above indicates that Akt, ERK1/2, and PKA pathways inhibit Bad function as well as triggering prosurvival signaling pathways after cerebral ischemia.¹⁴

Lastly, another family of regulatory proteins are the endogenous inhibitors of apoptosis (IAPs), which act by inhibiting caspases-3 and -9. Levels of X-linked IAP and the number of XIAP-positive cells show a strictly time- and region-dependent

evolution in cerebral ischemia, suggesting that this IAP plays a role in regulation of apoptosis in this setting.¹⁷ Transgenic mice overexpressing human XIAP undergo reduced caspase-3 activation after stroke and thus have reduced brain damage.⁴

Conclusions

It has become increasingly clear that there are many complex signaling pathways involved in apoptosis after cerebral ischemia. Although some inroads have been made in identifying specific molecular pathways involved in apoptosis after stroke, in the long-term we will require an integrated understanding of the multifaceted molecular processes that contribute to apoptosis to identify relevant novel stroke therapies.

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Disclosures

None.

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