Mitochondrial Targets for Stroke
Focusing Basic Science Research Toward Development of Clinically Translatable Therapeutics

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Background and Purpose—Stroke is a major cause of death and disability, and it is imperative to develop therapeutics to mitigate stroke-related injury. Despite many promising prospects, attempts at translating neuroprotective agents that show success in animal models of stroke have resulted in very limited clinical success.

Summary of Review—This review discusses reasons for the lack of translational success based on the therapeutic targets tested and the pathophysiology of stroke. New recanalization therapies and alternative therapeutic strategies are discussed concerning mitochondria-mediated cell death. Mitochondrial death-regulation pathways are divided into 3 categories: Upstream signaling pathways, agents that target mitochondria directly, and downstream death-execution effectors. The apoptosis signal-related kinase/c-Jun–terminal kinase pathway is used as an example to provide rationale as to why inhibiting signaling pathway upstream of mitochondrial dysfunction is a promising therapeutic approach. Finally, the mechanisms of autophagy and mitochondrial biogenesis are discussed in relation to stroke.

Conclusions—Increasing evidence suggests that reperfusion is necessary for improved neurological outcomes after stroke. Development of improved recanalization methods with increased therapeutic windows will aid in improving clinical outcome. Adjunct neuroprotective interventions must also be developed to ensure maximal brain tissue salvage. Targeting prodeath signaling pathways upstream of mitochondrial damage is promising for potential clinically effective treatment. Further understanding of the roles of equilibrium of autophagy and mitochondrial biogenesis in the pathogenesis of stroke could also lead to novel therapeutics. (Stroke. 2009;40:00-00.)

Key Words: cerebral ischemia ▪ therapeutics ▪ neuroprotection ▪ mitochondria
Mitochondrial Stroke Targets

Mitochondria are essential organelles involved with oxidative phosphorylation, calcium homeostasis, reactive oxygen species (ROS) management, and PCD. Convergence of a number of cell death pathways emanating from membrane receptors, the cytosol, nucleus, lysosome, and endoplasmic reticulum on the mitochondria results in mitochondrial destabilization. A common consequence of these death pathways is damage to the mitochondria, resulting in mitochondrial membrane permeabilization (MMP). Indeed, a number of assays have been developed to measure MMP as an indicator of cytotoxicity.

Mitochondrial membrane destabilization results in the release of mitochondrial components (ie, cytochrome c and apo-
sis-inducing factor), which in turn initiate the caspase-dependent and-independent intrinsic PCD pathways.

The effectors of mitochondria-related PCD can be divided into 3 categories: (1) downstream mitochondrial death effectors; (2) agents that directly target and destabilize mitochondrial membranes; and (3) upstream signaling mechanisms. Identifying the category of mitochondria-related PCD is critical for focusing the search on targets that will provide optimal protection against ischemia. The following section will discuss the 3 categories and their therapeutic potential.

**Downstream Mitochondrial Death Effectors**

Perturbation of the mitochondrial membrane results in release into the cytosol of cytotoxic molecules such as cytochrome c and apoptosis inducing factor (AIF) initiating caspase-dependent and-independent forms of cell death, respectively. In caspase-dependent cell death, released cytochrome c activates the caspase cascade, causing cleavage of many proteins, DNA damage, and ultimately cell death (for review see reference 1). Other mitochondrial proteins are also released to negatively regulate the endogenous inhibitors of caspases such as X-linked inhibitor of apoptosis (XIAP). Caspase-independent cell death occurs via calpain- or poly (ADP-ribose) polymerase-1 (PARP1)-mediated AIF release from the mitochondria, with subsequent translocation to the nucleus.17

Attempts at inhibiting these downstream mitochondrial death effectors have been successful at producing robust neuroprotection. A caveat is that neuroprotection as measured by both decreased infarct volume and neurobehavioral recovery has been assessed only after brief periods of reperfusion. Examining a recent review analyzing the neuroprotection afforded by downstream mitochondrial death effectors reveals that neuroprotection was assessed only between 24 hours to 1 week after insults of neonatal hypoxia/ischemia and focal and global ischemia in adult animals (see Table 1 in Galluzzi et al17). Assessment included caspase inhibition, XIAP overexpression, and protein transduction domain (PTD)-fused XIAP, HSP70 overexpression (which binds and sequesters AIF released from the mitochondria), AIF deletion, and peptide inhibition.

Two reasonable conclusions can be drawn from these data: (1) neuroprotection was not assessed longer than 1 week after the insult; or (2) the interventions were no longer neuroprotective at later times. Using the transient global ischemia model in rats, we designed an experiment to answer this question. Comparison of PTD-fused Apaf-1 interacting protein (AIP), an inhibitor of Apaf-1 and caspase-dependent PCD, and PTD-fused Bcl-xL was performed to determine whether inhibition of PCD at the mitochondria (Bcl-xL) or inhibition of a downstream death effector (AIP) could confer long-term neuroprotection. We found that although PTD-AIP administration decreased death of hippocampal CA1 neurons 4 days after ischemia, there was no neuroprotection or improved spatial learning or memory 60 days after ischemia. Alternatively, administration of Bcl-xL increased CA1 survival up to 60 days after ischemia and was associated with improved spatial learning and memory.18 Thus, inhibition of downstream effectors affords merely short-term protection, likely attributable to the presence of compensatory mechanisms of the mitochondria (ie, AIF release) or to mitochondria-independent mechanisms including genomic alterations and increased oxidative stress.

**Direct Targets of Mitochondria**

Direct effectors of mitochondrial membrane disruption act further upstream in the mitochondrial death decision pathway. The foremost proteins involved with mitochondrial impairment are the prodeath Bcl-2 family proteins including Bax, Bak, Bid, Bad, Bim, and PUMA, among others. The Bcl-2 multidomain proteins Bax and Bak directly cause mitochondrial membrane disruption via channel formation in the outer mitochondrial membrane. The BH3-only proteins Bid and PUMA act to facilitate Bax and Bak channel formation, whereas Bad and Bim inhibit prosurvival Bcl-2 and Bcl-xL.19

Analysis of a number of studies shows neuroprotection is afforded when either inhibition of prodeath Bcl-2 family proteins or increased prosurvival Bcl-2 family expression is performed (see Table 2 in Galluzzi et al17). However, none of these studies examined neuroprotection past 7 days. Examination of infarct volume 48 hours after focal ischemia shows that single gene deletion of prodeath Bcl-2 family proteins Bax, Bid, Bim, and PUMA produces limited neuroprotection. Even double knockout Bax/Bid mice provide just 48 hours of protection, as infarct volume is similar to controls at 14 days. However, triple knockout mice with deletions of Bax, Bid, and PUMA show prolonged neuroprotection up to 14 days (authors’ unpublished results, 2008). Single gene targeting of prodeath Bcl-2 family proteins is not sufficient to attenuate prodeath signaling, indicating that targeting 3 or more of this family of proteins is necessary to confer neuroprotection. Therefore, there is a functional redundancy in prodeath Bcl-2 family proteins, which is not surprising because different cell death stimuli can activate different prodeath Bcl-2 family proteins.20

Because there is a functional redundancy in the prodeath Bcl-2 family proteins, it is very probable that the broad insult of ischemia initiates numerous mechanisms activating multiple prodeath Bcl-2 family proteins. Therapeutically, one can look upstream to the natural inhibitors of the prodeath proteins. Prosurvival Bcl-2 and Bcl-xL sequester Bax and Bak, inhibiting oligomerization and mitochondrial membrane permeabilization. Recent studies have revealed that administration of PTD-fused Bcl-xL can provide robust neuroprotection 60 days after global ischemia18 and up to 8 weeks after neonatal hypoxia-ischemia.21

**Upstream Signaling Mechanisms**

**c-Jun N-Terminal Kinase**

Using the reasoning that selecting targets further upstream provides neuroprotection superior to that of downstream targets, we consider signaling pathways upstream of mitochondria-induced neuronal death. An important kinase consistently activated after cerebral-ischemia is the MAP kinase c-Jun N-terminal kinase (JNK; for review see reference 22). Inhibition of JNK signaling has high therapeutic potential because of its diverse mechanisms of inducing PCD.

Activation of JNK signaling directly and indirectly regulates prodeath Bcl-2 family proteins to cause mitochondrial membrane disruption. JNK phosphorylates the scaffolding...
protein 14-3-3, causing release and subsequent translocation of Bax to mitochondria.\textsuperscript{23} Bax activation is further induced by JNK-mediated phosphorylation of Bad and Bim, resulting in inhibition of prosurvival Bcl-xL and Bcl-2. Another consequence of JNK signaling is alterations in transcription of the transcription factor c-Jun. Activation of c-Jun promotes upregulation of Fas, Bim, and TNF\textsubscript{R} while decreasing expression of prosurvival Bcl-xL.\textsuperscript{24} Thus, JNK can induce neuronal death directly through mitochondrial death pathways, and alterations in the genomic response can shift gene expression toward production of prodeath mediators.

Importantly, the time frame for JNK activation coincides with the optimal time for maximal therapeutic potential. Elevated JNK activity begins at 6 hours and continues to 3 days in hippocampal CA1 neurons after global ischemia\textsuperscript{25} and between 0.5 and 24 hour following transient focal ischemia\textsuperscript{26} in rats. Indeed, compelling evidence has shown that inhibition of JNK using intracerebroventricular injection of the pharmacological inhibitor SP600125\textsuperscript{23} or the small peptide inhibitor D-JNKI-1\textsuperscript{26} provides robust neuroprotection after 60 or 30 minutes of focal ischemia, respectively. Long-lasting neuroprotection and neurobehavioral recovery are realized up to 14 days of reperfusion when D-JNKI-1 is administered 6 hours after ischemia.\textsuperscript{26} Remarkably, recent evidence also shows D-JNKI-1 administered i.v. 3 hours after 90-minute focal ischemia resulted in improved sensorimotor and cognitive outcome up to 10 days.\textsuperscript{27} In sum, this neuroprotective agent demonstrates robust and long-lasting neuroprotection and neurobehavioral recovery, and it can concurrently be administered systemically at delayed times. This is an exciting prospect for realizing clinical neuroprotection.

**Apoptosis Signal-Regulating Kinase**
Keeping with the rationale that targeting upstream effectors of neuronal death has greater potential to afford long-lasting neuroprotection, we consider targeting the MAP kinase kinase kinase upstream of JNK activation, apoptosis signal-regulating kinase 1 (ASK1). Oxidative stress, endoplasmic reticulum (ER) stress, calcium overload, and TNFR receptor (TNFR) stimulation—cytotoxic stresses that are implicated in ischemic damage—can all activate ASK1.\textsuperscript{28}

Another important factor involved with ischemia, PARP1, can enhance and be activated by ASK1/JNK signaling. Initially after ischemia, oxidative stress causes DNA damage activating PARP1 (for review, see\textsuperscript{29}). Activation of PARP1 can stimulate JNK via receptor-interacting protein 1 (RIP1) and TNF receptor–associated factor 2 (TRAF2) activation of ASK1. Activated JNK, in turn, can enable PARP1 activation,\textsuperscript{30} creating a feed-forward cycle. Thus, oxidative damage immediately causes ASK1 activation in the cytosol leading to JNK activation, mitochondrial damage with release of downstream death effectors, and increased ROS production. At the same time, oxidative stress causes DNA damage, PARP1 activation, and depletion of NAD. Depletion of NAD, an ATP precursor, results in increased ATP demand and further ROS production. PARP activation can also induce ASK1/JNK signaling, which can further perpetuate its own activity. Stresses occurring at later times after injury such as ER stress and inflammation-activated TNFR also stimulate ASK1/JNK, propagating PCD after a lethal ischemic insult (Figure 1). Thus, ASK1 is an attractive therapeutic target because of its extensive role in enacting and perpetuating cell death. The kinase domain of ASK1 is also evolutionarily conserved,\textsuperscript{28} facilitating development of small peptide inhibitors that have clinical potential.

**Future Targets for Stroke-Therapy Development**
The previous sections provide an argument for targeting upstream effectors of mitochondrial death pathways based on extensive mechanistic information. Burgeoning evidence also implicates the regulatory processes of mitochondrial biogenesis and autophagy in the pathogenesis of stroke. The next section discusses these topics in relation to the current information on their role in stroke and how targeting these processes may lead to alternative therapeutics.

**Autophagy**
Ischemic injury causes extensive and progressive damage to mitochondria, and the prevailing mechanism for eliminating damaged mitochondria is autophagy. Autophagy is a highly regulated process that breaks down organelles and macromolecules through lysosomal degradation and is essential for maintenance of intracellular homeostasis. It serves as a survival mechanism during times of limited nutrients, as macromolecules are recycled for ATP generation and nascent macromolecule synthesis. Autophagy can also cause autophagic or type II PCD, which is in contrast to type I PCD, or more classical caspase- and AIF-dependent PCD (for review see reference\textsuperscript{31}).

The role of autophagy after cerebral ischemia is beginning to be elucidated. An ischemic insult causes oxidative stress that damages multiple intracellular targets. Thus, efficient clearance of damaged organelles and macromolecules would be protective. In contrast, uncontrolled autophagy would lead to progressive digestion of affected neurons and neuronal death.

Genetic deletion of essential autophagy genes Atg 5 and 7 in mice results in neurodegeneration, suggesting that autophagy is important for normal neuronal function.\textsuperscript{32} Evidence of autophagy-induced PCD is also found in ischemia-effected neurons. Abrogation of Atg7 expression resulted in hippocampal CA1 neuron protection after neonatal hypoxic-ischemia,\textsuperscript{33} and inhibition of autophagy using pharmacological inhibitors reduced infarct volume after permanent focal ischemia.\textsuperscript{34} Beclin-1, a protein involved in autophagosome formation, is also upregulated after ischemia.\textsuperscript{32} Beclin-1 may be activated in ischemia via JNK inhibition of Beclin-1 binding protein, Bcl-2, allowing for autophagy activation.\textsuperscript{35} Thus, autophagy can be viewed as a double-edged sword; it is protective when activated by mild physiological stressors, but it can be detrimental to neuronal survival because of overactivation caused by a severe pathological stress such as ischemia (Figure 2).

**Mitochondrial Biogenesis**
Mitochondria are important for cellular homeostasis. However, mitochondria are not static organelles. Fluctuating homeostatic demands and inherent production of ROS by mitochondria cause progressive damage and require dynamic regulation of turnover,
content, function, and number. This is especially essential for proper function of postmitotic neurons.

Information is limited regarding the role of mitochondrial biogenesis in neurons. Initially, focus has been directed at transcriptional regulation of mitochondrial biogenesis. Neurodegeneration caused by oxidative stress is increased in a ROS-mediated manner in mitochondrial transcription factor peroxisome proliferator–activated receptor coactivator (PGC) 1α null mice. Hypoxic preconditioning stimulates neuronal nitric oxide synthase (nNOS)-dependent upregulation of PGC-1α expression. Oxidative stress also causes extensive mitochondrial fission, an event that precedes neuronal death. Interestingly, neuronal death can be abrogated by overexpression of the mitochondrial fusion protein mitofusin 2. This suggests that tight regulation of fission and fusion is important for neuronal viability. Finally, a recent study demonstrates that hypoxia-ischemia induces mitochondrial biogenesis. After hypoxia, increases are seen in mitochondrial DNA, total mitochondrial number, expression of the mitochondrial transcription factors downstream of PGC-1α (nuclear respiratory factor 1 and mitochondrial transcription factor A), and the mitochondrial protein HSP60. This is an exciting finding that suggests mitochondrial biogenesis is a novel endogenous neuroprotective response.

In summary, after a lethal ischemic insult, mitochondria are damaged, and autophagy is induced to remove damaged organelles. However, maintenance of energy levels and zinc and calcium homeostasis is necessary for neuronal function. Under stress, mitochondrial biogenesis be-
Mitochondrial death signaling is essential for programmed cell death, and targeting upstream signaling mechanisms has the greatest potential for long-term neuroprotection, even with delayed administration. Research should also focus on the roles of mitochondrial biogenesis and autophagy in the pathogenesis of cerebral ischemia. Damaged mitochondria must be discarded, but the ischemic insult disrupts the delicate balance of biogenesis and autophagy. Therefore, a multi-pronged strategy is needed, aimed at enhancing biogenesis, regulating autophagy, and inhibiting the deleterious actions of signaling pathways detrimental to mitochondrial function. Delineation of the key mediators of autophagy and mitochondrial biogenesis will provide novel targets for therapeutic development to enhance the beneficial effects of mitochondrial preservation.

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**Disclosures**

None.

**References**


