

# Mitochondria and Neuronal Death/Survival Signaling Pathways in Cerebral Ischemia\*

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Apoptotic cell death pathways have been implicated in acute brain injuries, including cerebral ischemia, brain trauma, and spinal cord injury, and in chronic neurodegenerative diseases. Experimental ischemia and reperfusion models, such as transient focal/global ischemia in rodents, have been thoroughly studied and suggest the involvement of mitochondria and the cell survival/death signaling pathways in cell death/survival cascades. Recent studies have implicated mitochondria-dependent apoptosis involving pro- and anti-apoptotic protein binding, the release of cytochrome *c* and second mitochondria-derived activator of caspase, the activation of downstream caspases-9 and -3, and DNA fragmentation. Reactive oxygen species are known to be significantly generated in the mitochondrial electron transport chain in the dysfunctional mitochondria during reperfusion after ischemia, and are also implicated in the survival signaling pathway that involves phosphatidylinositol-3-kinase (PI3-K), Akt, and downstream signaling molecules, like Bad, 14-3-3, and the proline-rich Akt substrate (PRAS), and their bindings. Further studies of these survival pathways may provide novel therapeutic strategies for clinical stroke.

**KEY WORDS:** Mitochondria; oxidative stress; cerebral ischemia; neuronal death signaling; neuronal survival signaling; PI3-K/Akt.

## ISCHEMIC APOPTOSIS SIGNALING PATHWAY INVOLVING MITOCHONDRIA

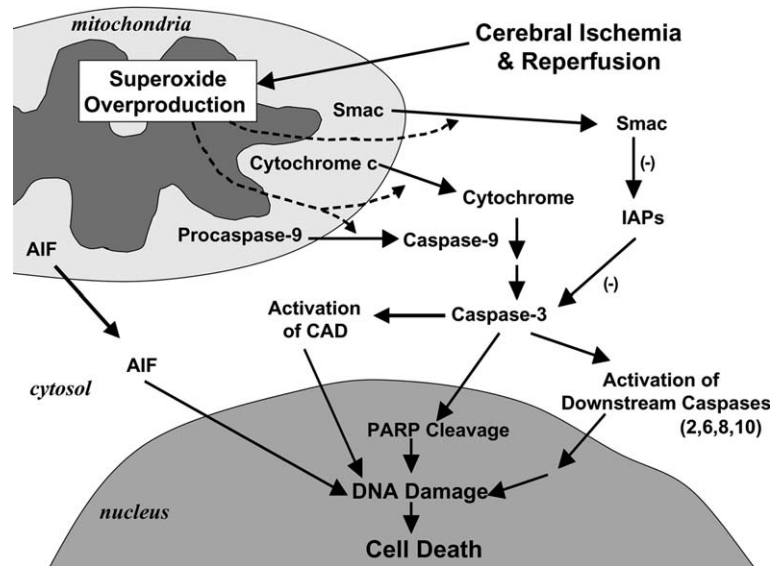
The cell death signaling pathway in mitochondria has recently been demonstrated in the ischemic brain with the release of mitochondrial cytochrome *c*, a water-soluble peripheral membrane protein of mitochondria and an essential component of the

mitochondrial respiratory chain (Fig. 1). Cytochrome *c* is translocated from mitochondria to the cytosolic compartment after transient focal cerebral ischemia (tFCI) in rats (1), in brain slices that are subjected to hypoxia-ischemia (2,3), and in vulnerable hippocampal CA<sub>1</sub> neurons after transient global cerebral ischemia (4). Mitochondria are known to be involved in both the necrosis and apoptosis pathways, which depend on severity of the insult or the nature of the signaling pathways (5–8). In most instances, severe cerebral ischemia renders the mitochondria completely dysfunctional for adenosine triphosphate production, which ensures necrotic cell death. In contrast, various *in vitro* studies demonstrate that cellular or biochemical signaling pathways involve mitochondria in apoptosis by releasing cytochrome *c* to the cytoplasm. Cytochrome *c*

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**Fig. 1.** Intrinsic mitochondria-dependent pathway of apoptosis in cerebral ischemia and reperfusion. AIF, apoptosis-inducing factor; CAD, caspase-activated DNase.

interacts with the CED-4 homolog, Apaf-1, and deoxyadenosine triphosphate, forming the apoptosome and leading to activation of caspase-9 (9–12). Caspase-9, which is an initiator of the cytochrome *c*-dependent caspase cascade, then activates caspase-3, followed by caspases-2, -6, -8 and -10 activation downstream (13). Caspase-3 also activates caspase-activated DNase and leads to DNA damage. In cerebral ischemia studies, caspases-3 and -9 have also been shown to play a key role in neuronal death after ischemia (14–16). Caspase-11 is also a critical initiator of caspases-1 and -3 activation, and caspase-11 knockout (KO) animals have shown reduced apoptosis after focal ischemia (17). Since caspase-11 is an upstream activator of caspase-1 in cytokine maturation, involvement of cytokines in apoptosis should also be considered after cerebral ischemia. The downstream caspases cleave many substrate proteins including poly(ADP-ribose) polymerase (PARP) (15,16,18). Substrate cleavage causes DNA injury and subsequently leads cells to apoptotic cell death, but excessive activation of PARP causes depletion of nicotinamide-adenine dinucleotide and adenosine triphosphate, which ultimately leads to cellular energy failure and death. Consistent with these notions, PARP KO mice showed decreased infarct after transient middle cerebral artery occlusion (MCAO) (19). A recent study has further demonstrated the role of PARP in the release of apoptosis-inducing factor from mitochon-

dria and subsequent translocation to the nucleus for DNA damage and apoptosis (20).

Conversely, there are proteins that can prevent caspase activation in the cytosol. The inhibitor-of-apoptosis protein (IAP) family suppresses apoptosis by preventing activation of procaspases and also by inhibiting enzymatic activity of active caspases (21,22). The second mitochondria-derived activator of caspase (Smac) is also released by apoptotic stimuli and binds IAPs, thereby promoting activation of caspase-3 (23). A recent study showed that mitochondrial release of cytochrome *c* and Smac preceded caspase activation after global ischemia, suggesting the importance of IAP inhibition as well as caspase activation (14). It is important to point out that these cell death signaling pathways are regulated by reactive oxygen species and the redox state of the cell during cerebral ischemia and reperfusion. Overexpressed cytosolic copper/zinc-superoxide dismutase (SOD1) in mice or rats significantly reduces the cell death signaling pathways involving cytochrome *c* and Smac release, activation of caspase-9 and caspase-3, binding of Smac and IAP, PARP activation and DNA fragmentation; whereas a deficiency in either SOD1 or mitochondrial manganese-SOD significantly exacerbates these cell death signaling pathways (4,5,14,24–30). These data suggest that both oxidative stress and the redox state play a role as a molecular switch for cell death or survival in apoptosis during cerebral ischemia and reperfusion.

### PRO- AND ANTI-APOPTOTIC PROTEINS THAT ARE ASSOCIATED WITH MITOCHONDRIAL-DEPENDENT APOPTOSIS/SURVIVAL

The Bcl-2 family proteins have one or more Bcl-2 homology domains and play a crucial role in intracellular apoptotic signal transduction by regulating permeability of the mitochondrial membrane (31). Although still controversial, many researchers believe that mitochondrial cytochrome *c* is released through the permeability transition pore (PTP), and that Bcl-2 family proteins directly regulate the PTP (32). Among these proteins, Bax, Bcl-X<sub>S</sub>, Bak, Bid and Bad are pro-apoptotic. They eliminate the mitochondrial membrane potential by affecting the PTP and facilitating cytochrome *c* release (33). Conversely, Bcl-2 and Bcl-X<sub>L</sub> function to conserve the membrane potential and block the release of cytochrome *c*. As expected, after focal cerebral ischemia, a decreased infarct was observed in transgenic mice that overexpress Bcl-2 (34) and in Bid KO animals (35), whereas, Bcl-2 KO mice showed an increased infarct (36). These findings, especially in the studies using pro-apoptotic/anti-apoptotic protein-transgenic/KO animals (Table I), suggest the importance of mitochondrial permeability regulation and Bcl-2 family proteins in ischemic cerebral injury.

Among the pro-apoptotic proteins, Bad, an important member of the Bax family, links the upstream cell survival signaling pathway and the downstream pathway to inactivate anti-apoptotic

Bcl-2 family proteins (37). *In vitro* studies show that Bad resides in an inactive complex with the molecular chaperone 14-3-3 via the phosphorylation of four serine residues (Ser-112, -136, -155, -170) (38). With apoptotic stimuli, Bad is dephosphorylated, dissociated from 14-3-3, and translocated to the outer membrane of the mitochondria, where it subsequently dimerizes with Bcl-X<sub>L</sub> and promotes mitochondrial cytochrome *c* release (38). Ser-155 residue is important for the direct interaction between Bad and Bcl-X<sub>L</sub> and its phosphorylation is regulated by several upstream signaling pathways. After cerebral ischemia, dephosphorylation and translocation of Bad from the cytosol to the mitochondria are observed and dimerization of Bad progresses with Bcl-X<sub>L</sub> in the early stages after MCAO (39). These results suggest the pivotal function of Bad in ischemic cell death.

### PHOSPHATIDYLINOSITOL-3-KINASE/AKT AS SURVIVAL SIGNALING PATHWAYS

There are several pathways to the inhibition of the pro-apoptotic function of Bad. Ras is thought to play a central role in signaling for growth factor-mediated resistance to apoptosis (40). Recent studies have shown that pharmacological blockade of Ras results in inhibition of the protective effects of ischemic preconditioning in primary cultures, and conversely, overexpression of Ras by transfection provides protection for cultured ischemic neurons

**Table I.** Transgenic and Knockout Studies of Pro-Apoptotic and Anti-Apoptotic Proteins

Study	Insult	Findings	Reference
Bid -/-	Transient MCAO	Decreased infarct (-67%)	(35)
Bcl-2 Tg	Permanent MCAO	Decreased infarct (-50%)	(34)
Bcl-2 Tg	Global ischemia	Decreased injury	(54)
Bcl-2 Tg	Permanent MCAO	No protection	(55)
Bcl-2 Tg	Permanent MCAO	Decreased injury	(56)
Bcl-2 -/+, -/-	Transient MCAO	Increased infarct	(36)
Bcl-X <sub>L</sub> Tg	Permanent MCAO	Decreased infarct (-21%)	(55)
Caspase-1 NM	Transient MCAO	Decreased infarct (-44%)	(57)
Caspase-1 NM	Permanent MCAO	Reduced injury	(58)
Caspase-1 -/-	Permanent MCAO	Reduced injury	(59)
Caspase-1 -/-	Transient MCAO	Decreased infarct	(60)
Caspase-11 -/-	Permanent MCAO	Reduced apoptosis	(17)
PARP -/-	Transient MCAO	Decreased infarct	(61)
PARP -/-	Transient MCAO	Decreased infarct in chronic stage	(62)
Fas NM	Transient MCAO	Decreased infarct	(63)
TNFR (p55 & p75) -/-	Transient MCAO	Increased injury	(64)
TNFR (p55 & p75) -/-	Transient MCAO	Increased injury	(65)
p53 -/+, -/-	Permanent MCAO	Decreased infarct (-27%, -15%)	(66)

Tg, transgenic; NM, negative mutant; TNFR, TNF-receptor- $\alpha$ .

(41). Ras can directly activate phosphatidylinositol-3-kinase (PI3-K), an upstream effector for activation of Akt. Akt is an initiator of the downstream pathways that inhibit the apoptotic pathways. Akt phosphorylates Bad and obviates its inhibitory effects on Bcl-X<sub>L</sub>, ultimately inhibiting the release of cytochrome *c* by blocking channel formation by Bax on the mitochondrial membrane (40). Akt also inhibits proteolytic activity of caspase-9 by phosphorylating it on Ser-196 (42). In addition, Akt can translocate into the nuclei and inactivate a proapoptotic member of the Forkhead family of transcription factors by phosphorylation, thereby inhibiting activation of the Fas pathway of apoptosis (43). Mitogen-activated protein kinase (MAPK) family members play a critical role in the regulation of cell growth, differentiation and cellular response to cytokines and stress (44). One MAPK-family member, extracellular signal-regulated kinase (ERK), has two isoforms (ERK1/2), which are constitutively expressed in the normal brain (45) and are activated by MAPK/ERK kinase 1/2. In this pathway, Ras recruits the main effector, Raf-1, to activate MAPK/ERK kinase 1/2 (46). Active ERK1/2 inactivates Bad through phosphorylation of 90-kDa ribosomal S6 kinases (47). Transforming growth factor- $\beta$ 1 has been shown to suppress Bad activity by phosphorylation of Bad at the Ser-112 site via activation of the ERK pathway in both

*in vivo* cerebral ischemia models and *in vitro* studies (48). Phosphorylation of ERK1/2 is involved in apoptosis and cell death after transient MCAO (49). Phosphorylation of the Ser-155 residue in Bad is regulated by protein kinase A (PKA) in studies *in vitro* (50). In rodent focal cerebral ischemia models, intraventricular injection of a PKA inhibitor, H89, effectively suppressed PKA activity (51) and dimerization of Bad/Bcl-X<sub>L</sub>, and subsequently, apoptotic cell death (39). This cumulative evidence suggests that Akt, ERK1/2 and PKA pathways inhibit the function of Bad as a cell survival signaling pathway after cerebral ischemia.

Besides Bad survival signaling, PI3-K/Akt is also involved in many other survival signaling pathways. One such pathway includes MDM2/p53 (52). Also, a novel proline-rich Akt substrate (PRAS) was recently detected and found to be involved in apoptosis. We have found that PRAS is phosphorylated by Akt in surviving cortical neurons and that phosphorylated PRAS (pPRAS) and the binding of pPRAS phosphorylated Akt (pPRAS/pAkt) to 14-3-3 (pPRAS/14-3-3) were altered, and their expression briefly decreased in mouse brains after tFCI. Liposome-mediated pPRAS cDNA transfection induced overexpression of pPRAS, promoted pPRAS/14-3-3, and inhibited apoptotic neuronal cell death after tFCI. The expression of pPRAS, pPRAS/pAkt and pPRAS/14-3-3 increased in nerve

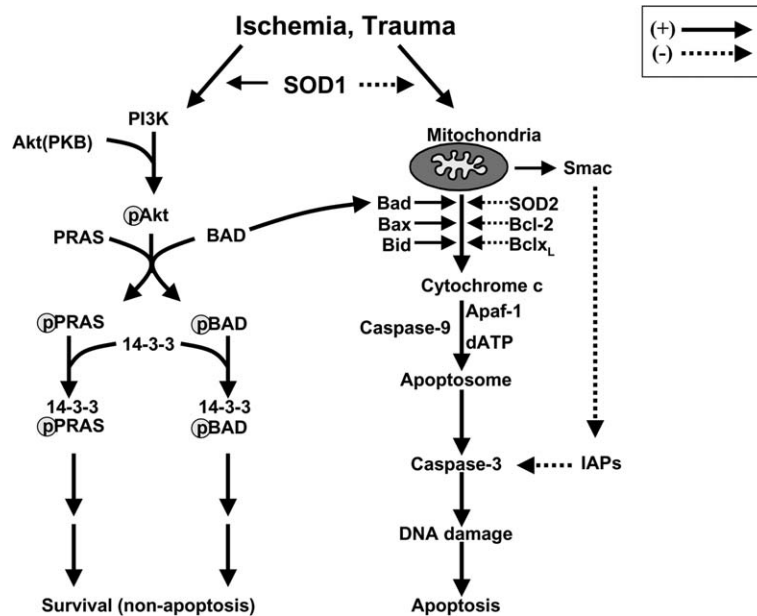


Fig. 2. Life and death signaling in ischemic neurons involving mitochondria and the PI3-K/Akt pathway. PKB, protein kinase B; SOD2, manganese-superoxide dismutase.

growth factor (NGF)-treated mice, but decreased with inhibition of PI3-K and the NGF trkA receptor after tFCI. These results suggest that PRAS phosphorylation and its interaction with pAkt and 14-3-3 might play an important role in neuroprotection mediated by NGF in anti-apoptotic neuronal cell death after tFCI. Further studies have also shown that oxidative stress is also involved in modulating the expression of pPRAS, pPRAS/pAkt, and pPRAS/14-3-3 binding (53), again suggesting that the PI3-K/Akt survival signaling pathway is upregulated by SOD1 overexpression (Fig. 2).

We now propose that mitochondria and the PI3-K/Akt signaling pathway are the determinants controlling pro-apoptosis and anti-apoptosis in ischemic neurons during stroke. Further studies of the survival signaling pathways may provide novel therapeutic strategies for clinical stroke.

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