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Oxidative Stress and Neuronal Death/Survival Signaling in Cerebral Ischemia

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Abstract

It has been demonstrated by numerous studies that apoptotic cell death pathways are implicated in ischemic cerebral injury in ischemia models in vivo. Experimental ischemia and reperfusion models, such as transient focal/global ischemia in rodents, have been thoroughly studied and the numerous reports suggest the involvement of cell survival/death signaling pathways in the pathogenesis of apoptotic cell death in ischemic lesions. In these models, reoxygenation during reperfusion provides oxygen as a substrate for numerous enzymatic oxidation reactions and for mitochondrial oxidative phosphorylation to produce adenosine triphosphate. Oxygen radicals, the products of these biochemical and physiological reactions, are known to damage cellular lipids, proteins, and nucleic acids and to initiate cell signaling pathways after cerebral ischemia. Genetic manipulation of intrinsic antioxidants and factors in the signaling pathways has provided substantial understanding of the mechanisms involved in cell death/survival signaling pathways and the role of oxygen radicals in ischemic cerebral injury. Future studies of these pathways could provide novel therapeutic strategies in clinical stroke.

Index Entries: Cerebral ischemia; oxidative stress; superoxide dismutase; mitochondria; stroke; oxygen radicals; survival signaling; apoptosis; PI3-kinase; Akt.

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Introduction

It has long been known that reactive oxygen radicals play important roles in the pathogenesis of various neurological disorders, such as ischemia, trauma, and degenerative disease. They damage cellular macromolecules, such as lipids, proteins, and nucleic acids, and lead to cell injury and death *(1,2).* In addition to these direct injuries, recent studies have shown that oxygen radicals are also involved in cell death/survival signaling pathways *(1,3–5).* Recent studies suggest that apoptotic cell death occurs in vivo in cerebral ischemia models *(1,6).* Experimental ischemia and reperfusion models, such as transient focal/global ischemia in rodents, have been thoroughly studied and the cumulative evidence suggests the involvement of cell survival/death signaling pathways in the pathogenesis of apoptotic cell death in ischemic lesions *(7–11).* In these models, reoxygenation during reperfusion provides a substrate for numerous enzymatic oxidation reactions *(1,2)* and for mitochondrial oxidative phosphorylation and the produced reactive oxygen species. In this review, the mechanisms of cell death/survival signaling pathways after ischemia and the involvement of oxygen radicals in these pathways will be discussed.

Oxidative Stress as a Molecular Switch for Ischemic Cell Death/Survival Signaling

Generation of Oxygen Radicals and Clearance Pathways

Many studies have shown that reactive oxygen radicals play important roles in cerebral infarction and apoptosis in cerebral ischemia and reperfusion and in the pathophysiology of various neurological disorders *(1,2,12,13).* In the cerebral ischemia and reperfusion models, cerebral blood flow is reduced by occluded vessels in brain regions that are supplied with oxygen. Reoxygenation during reperfusion provides a substrate for numerous enzymatic oxidation reactions. Mitochondria produce superoxide anion radicals and hydrogen peroxide (H_2O_2) under normal physiological conditions *(14).* These constantly produced reactive oxygen species (ROS) are scavenged by superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase. SOD, specifically, processes superoxide anions and produces H_2O_2, which is then detoxified by catalase or GSH-Px, and, finally, changed to water and oxygen. Hydroxyl radicals (^{-}OH) can be generated from H_2O_2 through the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow \bullet HO + Fe^{3+} + {}^{-}OH$). Other small molecular antioxidants, including glutathione (GSH), ascorbic acid, and α-tocopherol, are also involved in the detoxification of free radicals. Reperfusion after ischemia causes overproduction of ROS in mitochondria, and consumption of endogenous antioxidants by these radicals could lead to a dramatic rise in intracellular ROS. It has been demonstrated in numerous studies that ROS are directly involved with cellular macromolecules such as lipids, proteins, and nucleic acids in oxidative damage in ischemic tissues, which leads to cell death. Recent studies have provided evidence that indirect signaling pathways mediated by ROS can also cause cellular damage and death in cerebral ischemia and reperfusion.

Antioxidant Enzymes and Studies Using Transgenic and Knockout Animals

Superoxide dismutases are specific antioxidant enzymes that detoxify superoxide anions and produce H_2O_2. Three SODs, copper/zinc SOD (SOD1), manganese SOD (SOD2), and extracellular SOD (ECSOD), are major antioxidant enzymes based on cellular distribution and localization. SOD1 is a cytosolic enzyme with a level constituted at approx 0.1% of total proteins in mammalian cells. SOD2 is a mitochondrial enzyme, whereas ECSOD is an isoform that is localized in extracellular space, cerebrospinal fluid, and cerebral vessels *(15).*

All three SOD isoforms dismutate $O2^{-}$, forming H_2O_2, which is scavenged by catalase or GSH-Px at the expense of GSH. GSH is generated from oxidized GSH by GSH reductase in the presence of reduced nicotinamide-adenine dinucleotide phosphate. Other lipid peroxides are also scavenged by GSH-Px. SOD1 has been extensively used in experimental studies involving cerebral ischemia and reperfusion. Unfortunately, mixed and confusing results have been obtained when free nonmodified SOD1 was used. The extremely short half-life of SOD1 (6 min) in circulating blood and its failure to pass the blood–brain barrier and be taken up intracellularly make it difficult to use for enzyme therapy in cerebral ischemia *(16).* However, a modified enzyme with an increased half-life, polyethylene glycol-conjugated SOD1, has been successfully used to reduce infarct volume in rats subjected to focal cerebral ischemia *(17).* Liposome-entrapped SOD1 has an increased half-life (up to 4.2 h), blood–brain barrier permeability, and cellular uptake and has been proven to be an effective treatment for reducing the severity of ischemic and traumatic brain injuries *(18,19).*

Numerous studies using genetically modified mice that either overexpress or are deficient in SODs have been published (Table 1). In SOD1-overexpressing transgenic mice, a threefold increase in SOD1 activity has been observed in all brain regions in heterozygous mice, whereas in homozygous mice, a fivefold increase in SOD1 activity was achieved *(20).* In these mice, a 35% decrease in infarct volume was observed after permanent focal ischemia involving coagulation of the distal middle cerebral artery and occlusion of the bilateral common carotid artery *(21).* In global ischemia, SOD1 overexpression is neuroprotective, with a 50% reduction in hippocampal CA1 cell death *(22,23),* and this protection is probably partly the result of blocking of the mitochondrial pathway of apoptosis *(10).* The role of SOD1 in cerebral ischemia was further confirmed with the use of SOD1-deficient mice. These SOD1 knockout (KO) mice had increased cell death and edema after transient middle cerebral artery occlusion (MCAO) and global cerebral ischemia *(24–26).* In contrast, increased neuronal injury was observed in neonatal mice that overexpress SOD1 after hypoxia ischemia *(27,28).* The increased ischemic/hypoxic injury in these SOD1 transgenic mice was the result of the developmentally downregulated GSH-Px activity *(27,28).* The importance of mitochondrial production of oxygen radicals and the protective role of SOD2 after permanent cerebral ischemia have been demonstrated in SOD2 KO mice. These mutant mice show exacerbated infarct volume after permanent MCAO *(29),* and increased mitochondrial cytochrome-*c* release and subsequent DNA fragmentation after permanent focal cerebral ischemia *(30).* However, mice that overexpress SOD2 showed neuronal protection against oxidative stress after transient focal cerebral ischemia *(31).* The ECSOD level in the brain is much lower than in other organs, but recent studies have demonstrated that overexpression of this protein provides protection after focal and global ischemia, whereas KO animals showed a larger infarct after focal ischemia *(32–34).* Results from pharmacological trials and studies using transgenic/KO rodents provide strong evidence to support the importance of SODs and superoxide in the pathophysiology of ischemic brain injury.

Superoxide generated in mitochondria is processed by SOD as the first step in its clearance pathway. This step generates H_2O_2, which is still a harmful ROS. Catalase and GSH-Px catalyze the reduction of H_2O_2 to water and oxygen. Because constitutive catalase expression is at a low level in neurons compared with other organs *(35),* GSH-Px is especially important for detoxifying H_2O_2 after cerebral ischemia and reperfusion. There are at least five mammalian GSH-Px isoenzymes, and GSH-Px-1 is the most ubiquitous form and localizes in the cytosol and mitochondria in most tissue. Neuronal injury in GSH-Px-1 transgenic and KO mice has been examined after focal ischemia (Table 1).

Table 1
Transgenic and Knockout Studies of Superoxide Dismutases and Glutathione Peroxidase

Study	Insult	Findings	Ref.
Superoxide dismutase			
SOD1 +/–	Permanent MCAO	Decreased cortical infarct (–35%)	*21*
SOD1 +/–	Permanent MCAO	No protection	*74*
SOD1 +/–	Transient MCAO	Decreased infarct	*75*
SOD1 +/–	Transient MCAO	Sustained hsp70 mRNA expression	*76*
SOD1 +/–	Transient MCAO	Sustained c-fos mRNA expression	*77*
SOD1 +/–	Global ischemia	Induction of hsp70	*78*
SOD1 +/–	Transient MCAO	Decreased injury (–50%)	*79*
SOD1 +/–	Neonatal hypoxia	Increased injury in neonates	*27*
SOD1 +/–	Neonatal hypoxia	Increased injury in neonates	*28*
SOD1 +/–	Global ischemia	Decreased injury (–50%)	*22*
SOD1 +/–	Global ischemia	Decreased injury (–50%)	*29*
SOD1 +/–	Transient MCAO	Decreased DNA fragmentation	*80*
SOD1 +/–	Transient MCAO	Decreased cytochrome-*c* release	*7*
SOD1 +/–	Transient MCAO	Downregulation of nuclear factor-κB	*81*
SOD1 +/–	Transient MCAO	Decreased activation of activator protein-1	*82*
SOD1 +/–	Global ischemia	Decreased active caspase-3, caspase-9	*10*
SOD1 +/–	Transient MCAO	Decreased ERK activation	*8*
SOD1 +/–	Transient MCAO	Decreased Bad activation	*11*
SOD1 –/–	Transient MCAO	Increased infarct (+40%)	*25*
SOD1 –/–	Transient MCAO	Increased lesion size and edema	*26*
SOD1 –/–	Global ischemia	Increased cell death	*24*
SOD1 –/+, –/–	Permanent MCAO	No increase in infarct volume	*83*
SOD2 +/–	Transient MCAO	Decreased injury	*31*
SOD2 –/+	Permanent MCAO	Increased infarct (+66%)	*29*
SOD2 –/+	Permanent MCAO	Increased active caspase-9	*30*
SOD2 –/+	Transient MCAO	Increased cytochrome-*c* release	*56*
SOD2 –/+	Permanent MCAO	Increased superoxide production	*84*
ECSOD +/–	Transient MCAO	Decreased infarct (–28%)	*32*
ECSOD +/+	Global ischemia	Decreased injury (–48%)	*34*
ECSOD –/–	Transient MCAO	Increased infarct (+81%)	*33*
Glutathione peroxidase			
GSH-Px-1 +/+	Transient MCAO	Decreased infarct	*85*
GSH-Px-1 –/–	Transient MCAO	Increased apoptosis	*86*

Apoptosis Signaling Involving Mitochondria in Cerebral Ischemia

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 *(36)*, in brain slices that are subjected to hypoxia–ischemia *(37,38)*, and in vulnerable hippocampal CA_1 neurons after transient global cerebral ischemia *(9)*. Mitochondria are known to be involved in both the necrosis and apoptosis pathways, which depend on the severity of the insult or the nature of the signaling pathways

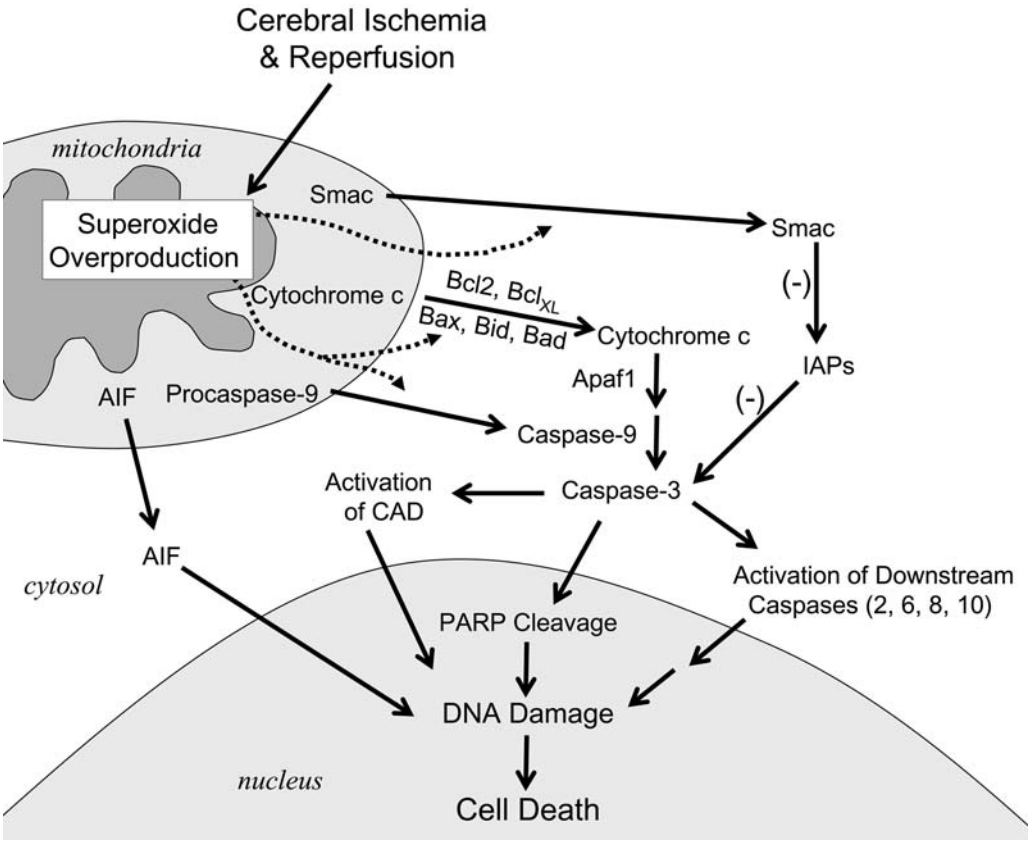

Fig. 1. Intrinsic mitochondria-dependent pathway of apoptosis in cerebral ischemia and reperfusion. AIF, apoptosis-inducing factor; CAD, caspase-activated DNase.

(7,39–41). 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 demonstrated that cellular or biochemical signaling pathways involve mitochondria in apoptosis by releasing cytochrome-*c* to the cytoplasm. Cytochrome-*c* interacts with the CED-4 homolog, Apaf-1, and deoxyadenosine triphosphate, forming the apoptosome and leading to activation of caspase-9 *(42–45).* Caspase-9, which is an initiator of the cytochrome-*c*-dependent caspase cascade, then activates caspase-3, followed by caspase-2, caspase-6, caspase-8, and caspase-10 activation downstream *(46).* Caspase-3 also activates caspase-activated DNase and leads to DNA damage. In cerebral ischemia studies, caspase-3 and caspase-9 have also been shown to play a key role in neuronal death after ischemia *(10,47,48).* Caspase-11 is also a critical initiator of caspase-1 and caspase-3 activation, and caspase-11 KO animals have shown reduced apoptosis after focal ischemia *(49).* Because 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) *(47,48,50).* 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 MCAO *(51).* A recent study has further demonstrated the role of PARP in the release of apoptosis-inducing factor from mitochondria and subsequent translocation to the nucleus for DNA damage and apoptosis *(52).*

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 by inhibiting the enzymatic activity of active caspases *(53,54).* The second mitochondria-derived activator of caspase (Smac) is also released by apoptotic stimuli and binds IAPs, thereby promoting activation of caspase-3 *(55).* 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 *(10).* It is essential to point out that these cell death signaling pathways are regulated by ROS and the redox state of the cell during cerebral ischemia and reperfusion. Overexpressed cytosolic 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, and PARP activation and DNA fragmentation, whereas a deficiency in either SOD1 or mitochondrial SOD2 significantly exacerbates these cell death signaling pathways *(1,4,7,9,10,22,26,29,30,56).* These data suggest that both oxidative stress and the redox state play a role as molecular switches for cell death or survival in apoptosis during cerebral ischemia and reperfusion.

Survival Signaling Involving Phosphatidylinositol 3-Kinase/Akt/Bad in Cerebral Ischemia

Cell survival pathways are the focus for clarifying the apoptotic neuronal cell death machinery. Serine/threonine kinases, such as Akt/protein kinase B, are key regulators of neuronal cell death and survival after cerebral ischemia *(57).* Akt functions as a major downstream target of phosphatidylinositol 3-kinase (PI3-K), and after phosphorylation, it phosphorylates some substrates on the serine or threonine residues, including glycogen synthase kinase-3, *Caenorhabditis elegans* DAF-16 transcription factor, Bad, phosphodiesterase 3B, adenosine triphosphate-citrate lyase, and the tuberous sclerosis complex-2 tumor suppressor gene product tuberin *(58–63).*

Akt phosphorylates Bad and obviates its inhibitory effects on Bcl-X_L, ultimately inhibiting the release of cytochrome-*c* by blocking channel formation by Bax on the mitochondrial membrane *(6).* Akt also inhibits proteolytic activity of caspase-9 by phosphorylating it on Ser-196 *(64).* 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 *(65).* 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 *(66).* One MAPK family member, extracellular signal-regulated kinase (ERK), has two isoforms (ERK1/2), which are constitutively expressed in the normal brain *(67)* 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 *(68).* Active ERK1/2 inactivates Bad through phosphorylation of 90-kDa ribosomal S6 kinases *(69).* Transforming growth factor-β1 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 *(70).* Phosphorylation of ERK1/2 is involved in apoptosis and cell death after transient MCAO *(8).* Phosphorylation of the Ser-155 residue in Bad is regulated by protein kinase A (PKA) in studies in vitro *(58).* In rodent focal cerebral ischemia models, intraventricular injection of a PKA inhibitor, H89, effectively suppressed PKA activity *(71)* and dimerization of Bad/Bcl-X_L, and subsequent apoptotic cell

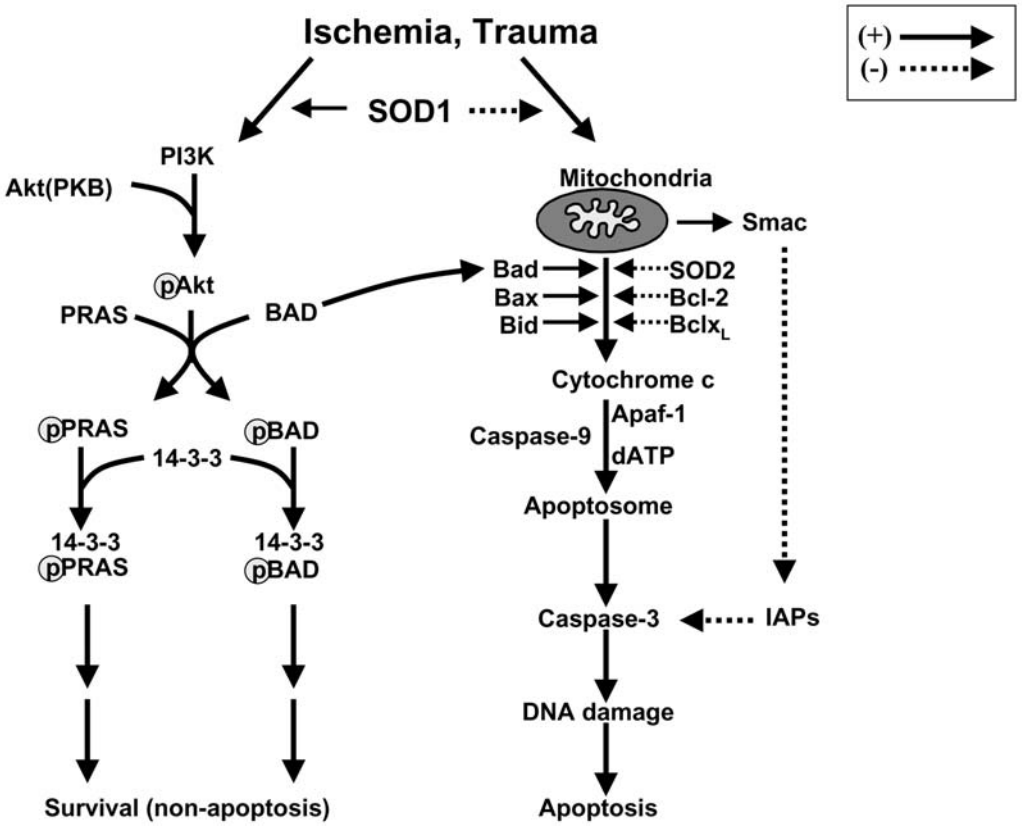

Fig. 2. Life and death signaling in ischemic neurons involving mitochondria and the PI3-K/Akt pathway. PKB, protein kinase B.

death *(11)*. 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.

In addition to Bad survival signaling, PI3-K/Akt is also involved in many other survival signaling pathways. One such pathway includes MDM2/p53 *(72)*. 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. Expression of pPRAS, pPRAS/pAkt, and pPRAS/14-3-3 increased in nerve growth factor-treated mice, but decreased with inhibition of PI3-K and the nerve growth factor 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 nerve growth factor in antiapoptotic neuronal cell death after tFCI. Further studies have also shown that oxidative stress is also involved in modulating the expression of pPRAS and pPRAS/pAkt, and pPRAS/14-3-3 binding *(73)*, 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 determinants for the control of proapoptosis and antiapoptosis in ischemic neurons during a stroke. Further studies of the survival signaling pathways could provide novel therapeutic strategies for clinical stroke.

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