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REVIEW

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# Apoptotic and antiapoptotic mechanisms in stroke

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**Abstract** Apoptosis is a form of programmed cell death that occurs in neurons during development of the nervous system and may also be a prominent form of neuronal death in chronic neurodegenerative disorders such as Alzheimer's and Parkinson's diseases. Recent findings also implicate apoptosis in neuronal degeneration after ischemic brain injury in animal models of stroke. Activation of both apoptotic and antiapoptotic signaling cascades occurs in neurons in animal and cell culture models of stroke. Apoptotic cascades involve: increased levels of intracellular oxyradicals and calcium; induction of expression of proteins such as Par-4 (prostate apoptosis response-4), which act by promoting mitochondrial dysfunction and suppressing antiapoptotic mechanisms; mitochondrial membrane depolarization, calcium uptake, and release of factors (e.g., cytochrome c) that ultimately induce nuclear DNA condensation and fragmentation; activation of cysteine proteases of the caspase family; activation of transcription factors such as AP-1 that may induce expression of "killer genes." Antiapoptotic signaling pathways are activated by neurotrophic factors, certain cytokines, and increases in oxidative and metabolic stress. Such protective pathways include: activation of the transcription factors (e.g., nuclear factor-κB, NF-κB) that induce expression of stress proteins, antioxidant enzymes, and calcium-regulating proteins; phosphorylation-mediated modulation of ion channels and membrane transporters; cytoskeletal alterations that modulate calcium homeostasis; and modulation of proteins that stabilize mitochondrial function (e.g., Bcl-2). Intervention studies in experimental stroke models have identified a battery of approaches of potential benefit in reducing neuronal death in stroke patients, including administration of antioxidants, calcium-stabilizing agents, caspase inhibitors, and agents that activate NF-κB. Interestingly, recent studies suggest novel dietary approaches (e.g., food restriction and supplementation with antioxidants) that may reduce brain damage following stroke.

**Key words** Calcium · Free radicals · Ischemia · Neurotrophic factor · NF-κB · Par-4

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## Introduction

Stroke is a major cause of disability and death worldwide. Brain damage following stroke results from a reduced blood supply to brain cells which drastically reduces their access to oxygen and glucose. Studies performed during the past 20 years have identified several key biochemical and cellular events that lead to ischemic neuronal degeneration (see Mattson and Mark 1996; Dirnagl et al. 1999, for reviews). Cellular ATP levels plummet rapidly following the onset of ischemia, which impairs the ability of membrane ion-motive ATPases to remove $Na^+$ and $Ca^{2+}$ from the cell. This results in membrane depolarization, which promotes activation of synaptic glutamate receptors. Excessive accumulation of extracellular glutamate further activates glutamate receptors, resulting in massive calcium influx through *N*-methyl-D-aspartate (NMDA) receptors and voltage-dependent calcium channels. Mitochondrial dysfunction occurs as the result of energy failure and disruption of cellular calcium homeostasis. Increased production of free radicals results from mitochondrial dysfunction, calcium overload, and activation of enzymes such as cyclooxygenase and nitric oxide synthase. Free radicals damage cellular proteins, DNA, and membrane lipids. A particularly important aspect of oxidative stress in neurons is membrane lipid peroxidation, which results in the generation of toxic aldehydes such as 4-hydroxynonenal that

impair the function of membrane ion-motive ATPases and glucose and glutamate transporters, thereby amplifying disruption of cellular calcium homeostasis (Mattson 1998). In addition to documenting that such alterations occur in various cell culture and animal models, intervention studies have demonstrated the efficacy of glutamate receptor antagonists, calcium-stabilizing agents, and antioxidants in reducing ischemic damage to neurons (Dirnagl et al. 1999).

A variety of valuable animal and cell culture models that mimic, in part, the pathogenic environment of neurons in the brains of stroke victims have been developed. Two widely employed models of ischemic brain injury are the transient global forebrain ischemia model, in which the entire blood supply to the brain is transiently interrupted, and the focal cerebral ischemia model, in which the middle cerebral artery is occluded, resulting in damage to cerebral cortex and striatum in that hemisphere (see Ginsberg and Busto 1989; Mhairi-Macrae 1992 for reviews). The focal model can involve either permanent or transient occlusion of the middle cerebral artery, with transient occlusion being generally accepted as the model that most closely duplicates stroke in human patients. In vitro models employ either dissociated cell cultures of hippocampal and cortical neurons, or hippocampal slices. The in vitro preparations can be subjected to glucose deprivation, hypoxia, excitatory amino acids, and oxidative insults (e.g., $Fe^{2+}$ and nitric oxide) to mimic specific aspects of the environment neurons encounter following stroke in vivo.

Neurons possess a variety of relatively unique features that must be kept in mind when studying mechanisms of ischemic brain injury. Neurons are, by definition, postmitotic and therefore largely irreplaceable once they die (but see Svendsen and Smith 1999 for recent evidence of the presence of neuronal precursor cells in the adult brain). Neurons also communicate with each other at highly specialized structures called synapses, in which a neurotransmitter released from the presynaptic cell activates receptors on the surface of the postsynaptic cell. Synapses are often located at a relatively large distance from the cell body, which is of considerable interest, since emerging data suggest synapses may be sites where neuronal apoptosis is often initiated (Mattson et al. 1998; Duan et al. 1999a). Finally, the glial cells that interact with neurons play important roles in modifying neuronal vulnerability to apoptosis. For example, astrocytes produce several different antiapoptotic growth factors (see below, Antiapoptotic signal transduction pathways that limit ischemic neuronal injury), while microglia (macrophages of the nervous system) produce neurotoxic substances such as nitric oxide and excitotoxins.

## Morphological and biochemical features of neuronal apoptosis

Details of the morphological and biochemical characteristics of apoptosis in nonneuronal cells are presented in other articles in this issue. For the most part, similar changes occur in neurons undergoing apoptosis. Thus, apoptotic neurons exhibit cell body shrinkage, formation of cell surface "blebs", and nuclear chromatin condensation and DNA fragmentation (Figs. 1, 2). The plasma membrane and organelles such as the mitochondria and endoplasmic reticulum (ER) remain intact during the cell death process. More unique to neuronal apoptosis is fragmentation of neurites (dendrites and axons), which occurs very early in the cell death process. Neurites are also damaged in neurons undergoing necrosis, but in the latter case ballooning and rapid disintegration of the neurites occurs, whereas in apoptotic neurons the fragmentation occurs with little swelling and over a longer time course. Apoptotic neurons are recognized by microglia and are engulfed; this clearance of apoptotic cells occurs without adversely affecting neighboring cells and without causing inflammation. Neurons dying by apoptosis are therefore often observed in isolation, with adjacent cells being unaffected. In many neuronal cell culture systems, microglia are not present and, in the latter settings, apoptotic cells will eventually undergo a secondary necrosis in which their plasma membranes and organelle membranes are disrupted. It is therefore important to recognize the existence of such secondary necrosis when studying neuronal apoptosis in culture systems.

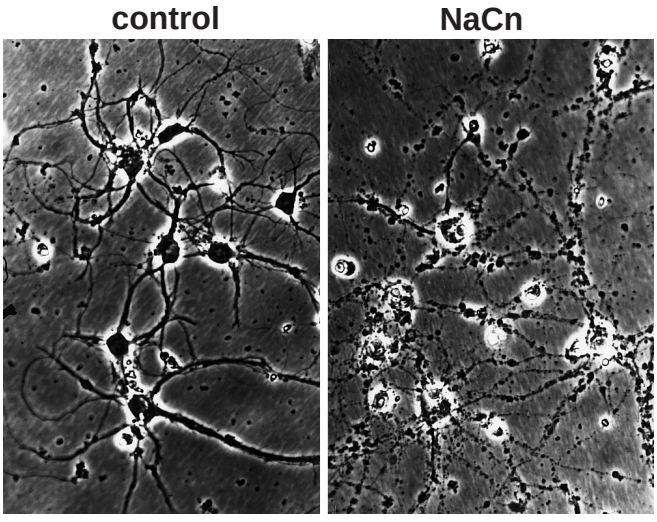


**Fig. 1** Examples of morphological features of cultured neurons undergoing apoptosis following chemical hypoxia. Phase-contrast micrographs of cultured rat hippocampal neurons that had been exposed to saline (*control*) or 0.5 mM *NaCn* for 16 h. Note that neurites are fragmented and the cell body is shrunken in neurons exposed to NaCn

Biochemical changes that may distinguish apoptosis and necrosis in neurons are beginning to be identified. Neurons undergoing apoptosis exhibit: rapid increases in prostate apoptosis response-4 (Par-4) protein levels (Guo et al. 1998; Chan et al. 1999; Duan et al. 1999a, 1999b); translocation of one or more members of the Bcl-2 protein family to mitochondrial membranes (Putcha et al. 1999); mitochondrial membrane depolarization and re-

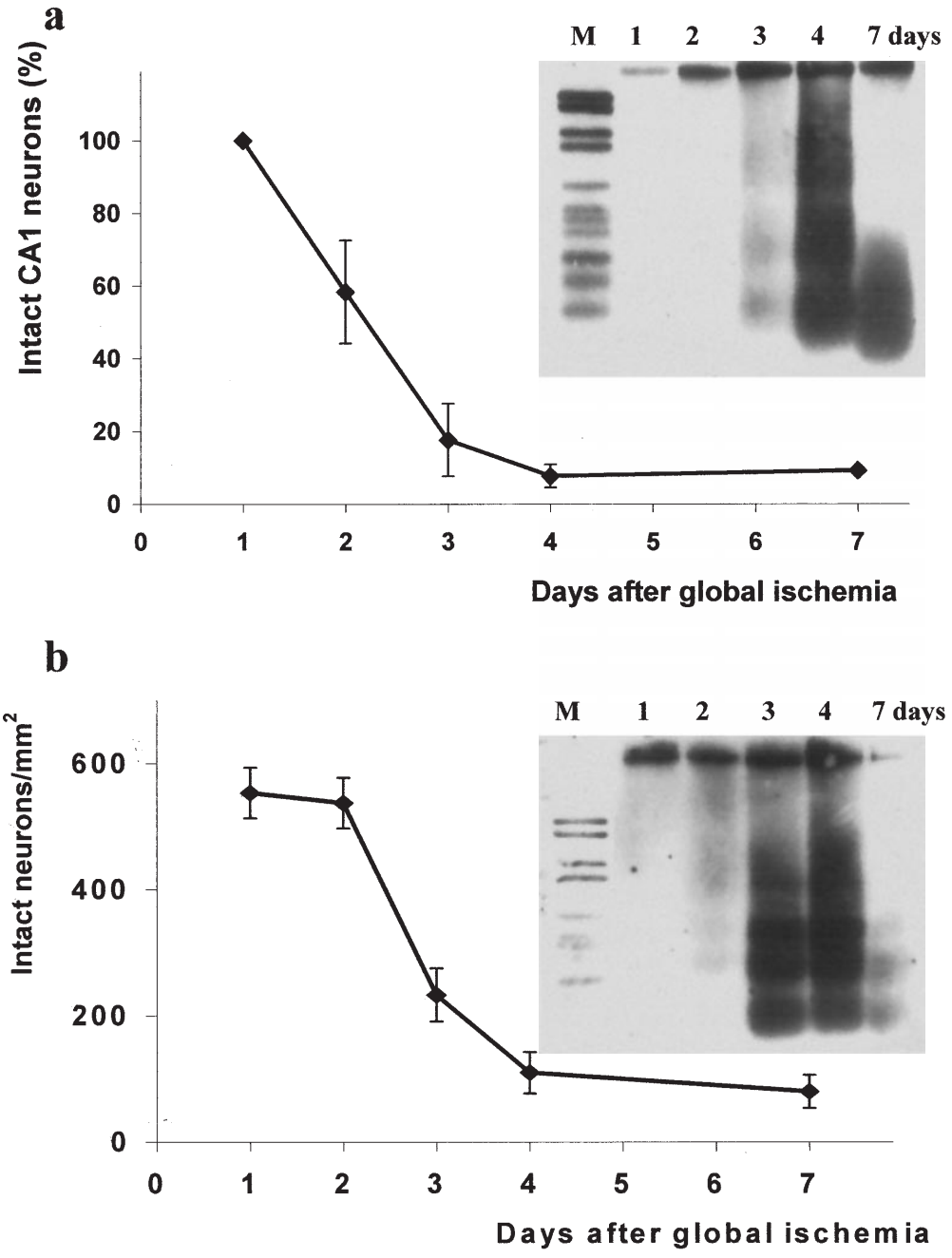


**Fig. 2a,b** Time courses of neuronal damage and associated DNA fragmentation in hippocampus and striatum after transient forebrain ischemia. Rat brains were removed 1, 2, 3, 4, and 7 days after 10 min of global ischemia induced by the occlusion of both common carotid arteries and reduction of mean arterial blood pressure to 40 mmHg. Neuronal damage in the CA1 subfield of the hippocampus (**a**) and the striatum (**b**) was quantified in four rats per time point by celestine blue and acid fuchsin staining. Data are presented as mean ± SD. For the detection of DNA fragmentation, the DNA was extracted from the brain tissue (QIAmp Tissue Kit, Qiagen, Hilden, Germany) and digoxigenin-labeled using the DIG oligonucleotide 3'-end labeling kit (Boehringer Mannheim, Germany). The labeled DNA fragments were seperated by electrophoresis, transferred onto a nylon membrane, and detected by an antidigoxigenin-alkaline phosphatase-CSPD system (Boehringer Mannheim, Germany). The blots presented demonstrate the time course of DNA fragmentation in the hippocampus (**a**) and the striatum (**b**) at *1, 2, 3, 4, and 7 days* after global ischemia. Note the correlation between delayed neuronal cell death induced by ischemia and the sustained detection of a DNA ladder typical for apoptosis in each brain region. (Modified from Zhu et al. 1998)

lease of cytochrome *c* into the cytoplasm (Neame et al. 1998): activation of one or more members of the caspase family of cysteine proteases (Chan and Mattson 1999); loss of plasma membrane phospholipid asymmetry which manifests in the presence of phosphatidylserine on the cell surface (a signal for engulfment by microglia); and nuclear DNA fragmentation (Kruman et al. 1997; Mattson et al. 1998). These changes are typically not observed in neurons undergoing necrosis, although, in certain experimental paradigms, neurons may exhibit both apoptotic and necrotic features (Nicotera and Lipton 1999).

Perhaps of equal importance to morphological and biochemical evaluation of apoptosis is the use of pharmacological manipulations known to prevent neuronal apoptosis but not necrosis. Antiapoptotic agents include: macromolecular synthesis inhibitors such as the protein synthesis inhibitor cycloheximide (Furukawa et al. 1997a); inhibitors of the caspase family of proteases such as zVAD-fmk and DEVD-fmk (Chan and Mattson 1999); and overexpression of antiapoptotic Bcl-2 family members such as Bcl-2 and Bcl-XL (Parsadanian et al. 1998; Wang et al. 1999).

## Molecular underpinnings of neuronal apoptosis

Cells undergoing apoptosis exhibit intriguing patterns of gene expression that involve the production of both pro-

and antiapoptotic proteins. Whether or not a neuron dies when subjected to potentially lethal insults is likely to depend upon whether anti- or proapoptotic pathways prevail. A rapidly increasing number of proapoptotic and antiapoptotic genes are being identified. Examples of proapoptotic genes are those encoding members of the caspase family of cysteine proteases, among which interleukin-1β converting enzyme (ICE) has been most widely studied. A pivotal role for caspase activation in neuronal apoptosis is underscored by the fact that peptide inhibitors of caspases (e.g., zVAD-fmk) can prevent apoptosis of neurons in a wide array of paradigms (Chan and Mattson 1999). Several members of the caspase family have been identified in mammalian cells and can be subdivided into three groups, based on homology with *C. elegans* homologues. Caspases are characterized by their ability to cleave proteins after aspartic acid residues and by the presence of the active cysteine in the middle of a conserved QACRG motif. Some of these proteases may be able to activate themselves or act on each other in a hierarchical cascade; for example, mature ICE can activate pro-ICE as well as caspase-3. There appear to be many different substrates for caspases, but it has not been established which substrates are of critical importance in the cell death process (Chan and Mattson 1999). Caspase substrates that are candidates for effectors of neuronal apoptosis include poly-ADP ribose polymerase (PARP), DNA-dependent protein kinase, U1-soluble nuclear RNA polymerase (U1-snRNP), spectrin, lamin A, actin, and protein kinase C. Although no single cleavage event may be critically important for death, the simultaneous cleavage of all these substrates (and probably others that are currently unknown) may ensure that death occurs in a rapid and efficient manner.

A protein identified as having antiapoptotic activity in many different cell types, including neurons, is Bcl-2, a mammalian homologue of the *C. elegans* antideath protein Ced-9 (Kroemer et al. 1997). Bcl-2 is one of a family of related proteins; some of the family members also have antiapoptotic properties (e.g., Bcl-Xl, Mcl-1), whereas others are proapoptotic (e.g., Bax, Bad, Bak, Bcl-Xs). Expression of Bcl-2 in cultured sympathetic neurons or PC12 cells prevents apoptosis induced by nerve growth factor (NGF) withdrawal (Guo et al. 1997). Bcl-2 also protects cultured neurons against apoptosis induced by amyloid β-peptide and oxidative insults (Kruman et al. 1997). Bcl-2 is expressed throughout the developing nervous system, but its expression declines in the adult central nervous system. The environmental factors affecting Bcl-2 expression are largely unknown, but probable candidates include neurotrophic factors and cytokines (see below, Antiapoptotic signal transduction pathways that limit ischemic neuronal injury).

## Roles of oxyradicals and calcium in neuronal apoptosis in stroke

The evidence that various reactive oxygen species (ROS) play roles in the neuronal apoptosis that occurs following stroke is compelling. In both transient global forebrain ischemia and focal ischemia rodent models, levels of oxidative damage to proteins, lipids, and DNA have been documented in the vulnerable neuronal populations (Carney et al. 1996; Chan 1996; Hayashi et al. 1999). Lipid peroxidation appears to play a particularly prominent role in neuronal apoptosis in experimental models relevant to the pathogenesis of stroke. The mechanism whereby membrane lipid peroxidation induces neuronal apoptosis appears to involve generation of an aldehyde called 4-hydroxynonenal, which covalently modifies membrane transporters (e.g., $Na^+$ ATPase, $K^+$ ATPase, glucose transporter, and glutamate transporter) thereby impairing their function (Keller et al. 1997; Kruman et al. 1997; Mark et al. 1997a, 1997b). Mitochondria-derived ROS appear to play a particularly important role in triggering changes that lead to neuronal apoptosis, because overexpression of mitochondrial superoxide dismutases (Mn-SOD) can prevent apoptosis induced by an array of insults, including ischemia (Keller et al. 1998). The antiapoptotic property of Bcl-2 is associated with its ability to suppress accumulation of ROS (Hockenbery et al. 1993), and Bcl-2 may act locally in membranes (particularly mitochondrial and plasma membranes) to suppress lipid peroxidation (Bruce-Keller et al. 1997). Overexpression of Bcl-2 in cultured neural cells results in resistance of the cells to oxidative stress-induced apoptosis (Kruman et al. 1997). Moreover, overexpression of Bcl-2 in cortical neurons via virus-mediated transfection reduces focal ischemic injury in vivo (Linnik et al. 1995), and transgenic mice overexpressing Bcl-2 exhibit increased resistance of neurons to ischemic injury (Kitagawa et al. 1998). Administration of antioxidants (e.g., vitamin E, propyl gallate, estrogens, and glutathione) to cultured neurons can protect them from apoptosis induced by insults relevant to ischemic brain injury, including exposure to glutamate, glucose deprivation, and $Fe^{2+}$ (Goodman et al. 1996; Mark et al. 1997a).

The sources of oxyradicals that are probably involved in neuronal apoptosis following cerebral ischemia are shown in Fig. 3. Mitochondria are the major intrinsic source of oxyradicals, with superoxide anion radical $O_2^{\cdot -}$ being constantly generated during the process of electron transport in mitochondria. Superoxide dismutases located in the mitochondria (Mn-SOD, also called SOD2) and cytoplasm (Cu/Zn-SOD or Zn-SOD, also called SOD1) convert $O_2^{\cdot -}$ to $H_2O_2$. $H_2O_2$ is not a free radical, but is a major source for generation of hydroxyl radical ($OH\cdot$) which is formed in the Fenton reaction catalyzed by $Fe^{2+}$. Other pathways for oxyradical production in neurons include interaction of nitric oxide ($NO\cdot$) with $O_2^{\cdot -}$ to form peroxynitrite, and $O_2^{\cdot -}$ production via activity of various oxygenases (e.g., cyclo-oxygenases, lipoxygenases, and heme oxygenase). An important oxyradical cascade leading to cell damage and death involves $OH\cdot$ attack at double bonds in membrane fatty acids, resulting an autolytic cascade called lipid peroxidation. Lipid peroxidation is probably a key event leading to disruption of ion homeostasis in several different paradigms of neuronal apopto-

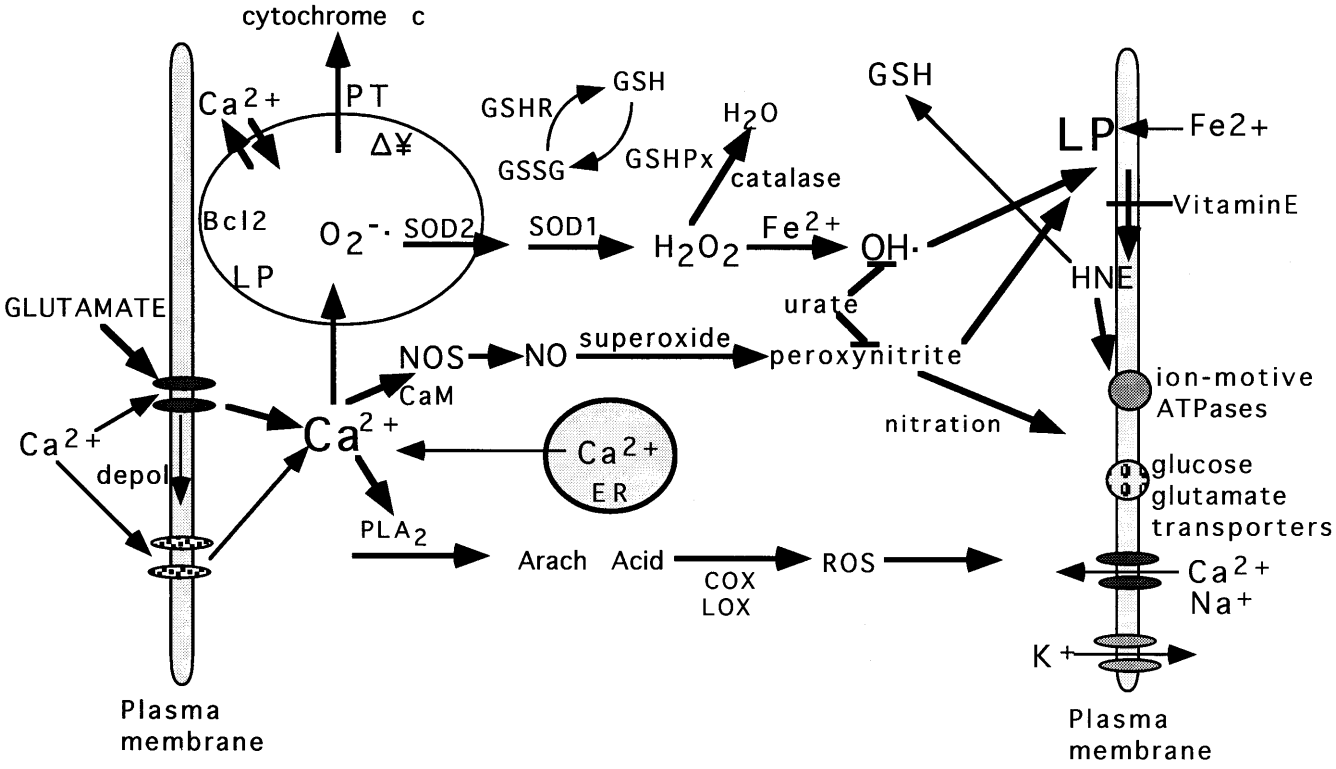


**Fig. 3** Mechanisms for the generation and detoxification of reactive oxygen species (*ROS*), and regulation of calcium homeostasis in neurons. The major endogenous source of oxyradicals in neurons is mitochondria wherein $O_2^{\cdot-}$ is generated during the electron transport process. Superoxide dismutases (*SOD1* and *SOD2*) convert $O_2^{\cdot-}$ to $H_2O_2$, which, in the presence of $Fe^{2+}$, generates OH·. Interaction of nitric oxide (*NO*) with $O_2^{\cdot-}$ results in the formation of peroxynitrite. Both OH· and peroxynitrite can induce membrane lipid peroxidation (*LP*) in the plasma membrane, and in mitochondrial and endoplasmic reticulum (*ER*) membranes. In addition, $Fe^{2+}$ (to which neurons are exposed following stroke) can induce LP. 4-Hydroxynonenal (*HNE*), which is generated when membrane lipids are peroxidized, binds to membrane transporters and ion channels and alters their activities. Glutathione (*GSH*) can bind and detoxify HNE. LP-induced impairment of the $Na^+/K^+$ ATPase, glucose transporter, and glutamate transporters results in membrane depolarization and excessive activation of glutamate receptors, resulting in excitotoxicity. LP also perturbs ion homeostasis in ER and mitochondria and compromises their important $Ca^{2+}$ sequestration functions. Antiapoptotic gene products such as Bcl-2 may act, in part, by suppressing LP in plasma, and mitochondrial and ER membranes. Elevation of $[Ca^{2+}]_i$, as occurs when glutamate receptors are activated, promotes LP by inducing NO· and $O_2^{\cdot-}$ production, as well as by activation of phospholipases ($PLA_2$), resulting in production of arachidonic acid (*Arach acid*), which is then acted upon by cyclooxygenases (*COX*) and lipoxygenases (*LOX*), resulting in $O_2^{\cdot-}$ production. See text for further discussion (*GSSG* glutathione disulfide)

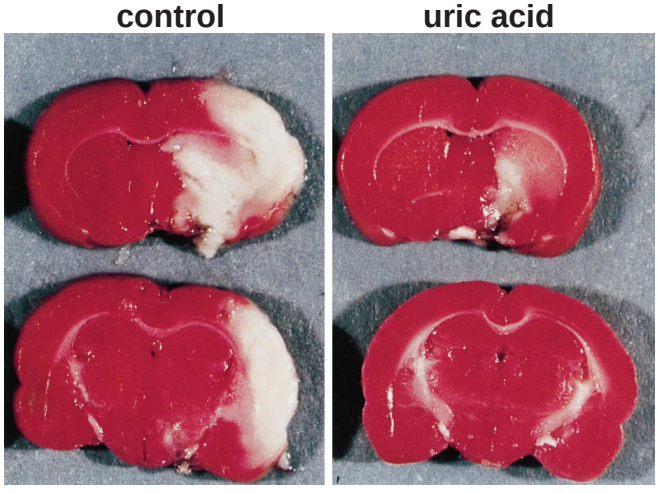


**Fig. 4** Uric acid, a scavenger of peroxynitrite and hydroxyl radical, reduces infarct size in a rat model of focal ischemic brain injury. Representative coronal brain sections from two rats that had been subjected to middle cerebral artery occlusion-reperfusion. One rat had been administered saline (*control*), while the other rat had been administered *uric acid* prior to ischemia. The *red staining* indicates healthy brain tissue and the *white* indicates damaged tissue. Note marked reduction in damage to cerebral cortex and underlying striatum in the rat treated with uric acid. (Modified from Yu et al. 1998)

sis (Kruman et al. 1997). Evidence that oxidative stress contributes to ischemic neuronal injury comes from studies demonstrating beneficial effects of antioxidants in animal models of stroke. For example, administration of uric acid (a scavenger of peroxynitrite and hydroxyl radical) to adult rats prior to middle cerebral artery occlusion-reperfusion markedly reduced damage to cerebral cortex and striatum and improved behavioral outcome (Fig. 4; Yu et al. 1998). Administration of vitamin E for 3 weeks prior to ischemia resulted in a marked decrease in numbers of apoptotic neurons in region CA1 as determined by electron-microscopic analysis (Tagami et al. 1999).

Calcium is a prominent second messenger in neurons, where it plays pivotal roles in many fundamental physio-

logical processes, including regulation of neurite outgrowth and synaptogenesis, neurotransmitter release, and synaptic plasticity. However, excessive and sustained increases in $[Ca^{2+}]_i$ can kill neurons. Calcium plays a central role in the death of neurons occurring in settings relevant to the pathogenesis of stroke (see Mattson and Mark 1996 for review). For example, calcium mediates death of cultured hippocampal and cortical neurons following exposure to hypoxia, hypoglycemia, and glutamate. Increased levels of mitochondrial calcium have also been linked to neuronal apoptosis in some systems and may play a pivotal role in mitochondrial membrane permeability transition and release of cytochrome *c* (Kluck et al. 1997; Budd 1998; Kruman and Mattson 1999). Calcium release from ER stores appears to make an important contribution to neuronal calcium overload in several different neurodegenerative conditions, including stroke (Mattson et al. 2000a). Evidence supporting the function of ER calcium release in stroke comes from studies showing that drugs that block calcium release from ER, including dantrolene (Tasker et al. 1998) and xestospongin (M. P. Mattson, unpublished data), protect neurons against injury in experimental stroke models.

## Evidence for neuronal apoptosis in experimental models of stroke

### Data from human patients

Very few studies have been aimed at establishing a role for neuronal apoptosis in brain injury following stroke in human patients. A major impediment to such studies is the inability to obtain postmortem tissue at poststroke time points that correspond to the time window when neuronal death is occurring. Thus, most stroke patients either die acutely or survive for relatively long time periods of months to years. However, in the few studies that have been performed, the data do suggest that neuronal apoptosis occurs in human stroke patients. One study employed a DNA end-labeling technique in parallel analyses of brain tissue from stroke patients and rats subjected to transient global forebrain ischemia (Guglielmo et al. 1998). They observed a similar temporal profile in appearance of TdT-mediated dUTP-biotin nick end labeling(TUNEL)-positive neurons following stroke in humans and in the rat model. A study of postmortem brain tissue from patients that suffered severe brain ischemia as the result of cardiac arrest showed that levels of PARP and Ku80 were increased (indicating DNA damage), particularly in regions that also contained TUNEL-positive neurons, including the CA1 region of hippocampus and deep layers of the cerebral cortex (Love et al. 1998). In a very recent study, 30 stroke patients were followed prospectively for 90 days, with clinical evaluation, radiological assessment, and measurements of levels of apoptosis-related proteins in cerebrospinal fluid (Tarkowski et al. 1999). The authors found that levels of soluble Fas/APO-1 were decreased during the entire study period and that levels of soluble Bcl-2 were decreased during the first 3 days following stroke onset. These findings suggest that levels of antiapoptotic proteins may be reduced following stroke and may thereby contribute to apoptotic death of neurons. Finally, analyses of carotid arteries of patients who underwent atherectomy because of their high level of atherosclerosis revealed increased levels of Bax immunoreactivity in vascular cells compared with controls (Konstadoulakis et al. 1998). The latter findings suggest that apoptosis may play a role in the kind of damage to cerebral vessels that places individuals at high risk of stroke.

### Data from animal models

The infarct resulting from focal cerebral ischemia following middle cerebral artery occlusion in rats and mice can be viewed as a sphere in which cells in the inner core die rapidly (within minutes to a few hours) by necrosis, whereas the cells in the outer "penumbral" regions die more slowly, over periods of days to weeks. Linnik and coworkers were among the first to provide evidence that neurons in the ischemic penumbra undergo apoptosis. They showed that many neurons in the ischemic cortex exhibited DNA strand breaks consistent with apoptosis within 7 days following middle cerebral artery occlusion (Linnik et al. 1993). In a subsequent study, they showed that, when neurons in the cerebral cortex were made to overexpress Bcl-2 (via virus-mediated transfection), they exhibited increased resistance to ischemia-induced death (Linnik et al. 1995). An example showing the time course of DNA fragmentation in tissue samples from hippocampus and striatum of rats following transient global forebrain ischemia is shown in Fig. 2. In both brain regions, the DNA fragmentation is delayed for several days following the ischemic insult.

Analyses of the role of mitochondrial alterations in neuronal death following focal cerebral ischemia provide further support for a major contribution of apoptosis to the pathophysiology of stroke. Infarct size following middle cerebral artery occlusion-reperfusion was significantly decreased in transgenic mice overexpressing mitochondrial Mn-SOD, indicating that mitochondrial superoxide production contributes to ischemic neuronal death (Keller et al. 1998). The latter study further showed that cultured neurons overexpressing Mn-SOD are resistant to apoptosis induced by insults relevant to stroke. Fujimura et al. (1998) demonstrated that cytochrome *c* redistributes from the mitochondrial fraction to the cytosolic fraction in cortical tissue following transient focal ischemia in rats.

Immunoblot and immunohistochemical analyses using antibodies against caspase substrates such as spectrin and actin have provided evidence that caspases are activated in neurons in the ischemic penumbra prior to their death. Caspase-8 is activated within 6 h of focal ischemia induced by permanent occlusion of the middle ce-

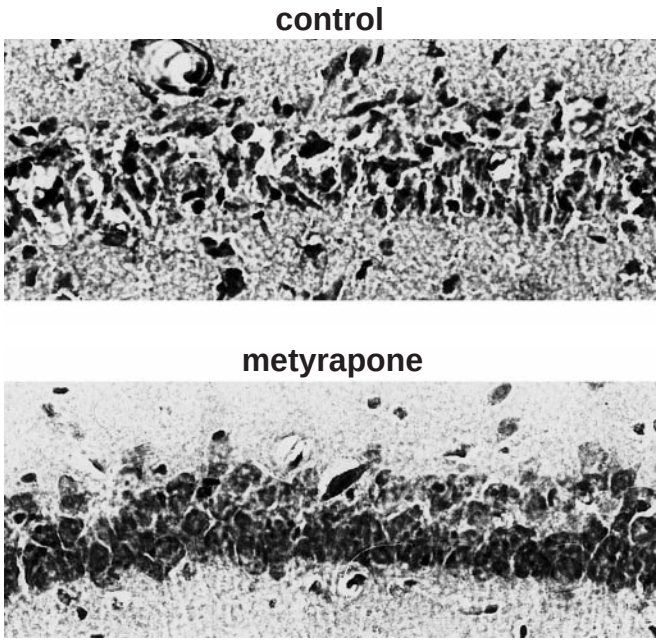


**Fig. 5** Metyrapone, an inhibitor of glucocorticoid production, protects CA1 hippocampal neurons against ischemic injury. Micrographs of Nissl-stained brain sections showing neurons in region CA1 of hippocampus in a saline-treated rat (*control*) and a rat that had been administered *metyrapone* prior to transient global forebrain ischemia. Note shrinkage and loss of CA1 neurons in the control rat, and greatly reduced damage and death of CA1 neurons in the metyrapone-treated rat. (Modified from Smith-Swintosky et al. 1996)

rebral artery in rats (Velier et al. 1999). Active caspase-8 was located mainly in pyramidal neurons of layer V of cerebral cortex. Active caspase-3 was observed in neurons in layers II and III, beginning 24 h after the onset of ischemia, and in microglia. Intraventricular administration of caspase inhibitors prior to or 6 h postreperfusion, resulted in a significant decrease in cerebral infarct size (Hara et al. 1997; Endres et al. 1998). The caspases that may mediate ischemic neuronal death probably include caspase-1 and caspase-3. Indeed, ischemic brain injury was decreased in mice lacking caspase-1 (Schielke et al. 1998).

The expression of genes that are believed to either promote or prevent neuronal apoptosis are altered in cells in the ischemic penumbra following focal cerebral ischemia. For example, levels of Fas and tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) increase in vulnerable brain regions following focal ischemia-reperfusion in rats (Martin-Villalba et al. 1999). The latter study also showed that Fas ligand induced apoptosis of cultured neurons, providing additional evidence for a role for Fas signaling in focal ischemic neuronal death .

Apoptotic cascades appear to contribute to the selective delayed death of CA1 hippocampal neurons following transient global forebrain ischemia. CA1 neurons shrink rather than swell in this model, consistent with apoptosis (Fig. 5). MacManus et al. (1993) showed that CA1 neurons exhibit nuclear DNA damage consistent with apoptosis, as assessed using a DNA end-labeling technique. Nitatori et al. (1995) provided further morphological evidence supporting a role for apoptosis in ischemic damage to CA1 neurons. They showed that both single-strand breaks and double-strand breaks occur between 48 and 72 h postreperfusion in the four-vessel occlusion ischemia model in rats (Jin et al. 1999). Opposing such evidence for neuronal apoptosis are data from electron-microscopic analyses that reveal evidence of necrotic death of CA1 neurons, but no evidence of typical apoptotic morphology, following transient global ischemia (Colbourne et al. 1999). Chen and coworkers (Chen et al. 1998) showed that caspase-3 activation precedes death of CA1 neurons, and administration of the broad-spectrum caspase inhibitor zVAD-fmk protects those neurons. Intraventricular administration of the pseudosubstrate caspase inhibitor *N*-tosyl-L-phenylalanyl chloromethyl ketone reduced damage to CA1 neurons following transient global ischemia in rats (Hara et al. 1998). Loss of CA1 neurons induced by transient global forebrain ischemia is preceded by caspase-3 activation, and overexpression of X chromosome-linked inhibitor of apoptosis protein (XIAP) via virus-mediated transfection prevented caspase-3 activation, death of CA1 neurons, and spatial memory deficits (Xu et al. 1999). Following transient global ischemia, caspase-9 is released from mitochondria and accumulates in nuclei of hippocampal CA1 neurons (Krajewski et al. 1999). In addition, transgenic mice overexpressing Bcl-2 exhibit increased resistance of hippocampal neurons to transient global forebrain ischemia (Kitagawa et al. 1998).

Increased expression of cell death-related genes in hippocampal neurons has been documented in studies of transient global forebrain ischemia in rodents. Examples include: (a) levels of p53 are markedly and persistently increased in vulnerable CA1 neurons, whereas levels of p53 only increase transiently in resistant CA3 neurons (McGahan et al. 1998); (b) expression of the DNA damage-sensing protein GADD-45 is increased in hippocampal neurons (Hou et al. 1997); (c) Bax levels increase following transient global ischemia, and the Bcl-2 interaction with Bcl-xL decreases, consistent with apoptosis-related changes in Bcl-2 family members (Antonawich et al. 1998).

Interestingly, steroid hormones have quite striking effects on neuronal injury in both focal ischemia and transient global forebrain ischemia models. For example, increased levels of glucocorticoids (adrenal steroids produced in response to physiological stress) enhance death of CA1 neurons following transient global ischemia and increase infarct size following focal ischemia in rats (Fig. 5; Smith-Swintosky et al. 1996). Conversely estrogens reduce ischemic brain injury in these rodent models (Sudo et al. 1997; Alkayed et al. 1998). Cell culture studies have shown that glucocorticoids can enhance and estrogens can prevent neuronal apoptosis following insults relevant to ischemic brain injury (Goodman et al. 1996; Mattson et al. 1997a). It is therefore likely that

these steroids have important effects on apoptotic signaling pathways in neurons following stroke.

### Data from cell culture models

Cell culture models of ischemic brain injury have elucidated the cellular and biochemical mechanisms that may lead to neuronal apoptosis in stroke. A typical approach is to expose cultures of relevant neuronal populations to agents that are believed to either play a direct role in the neuronal death process in vivo or activate a relevant cytodestructive pathway in the neurons. Two insults of direct relevance to stroke are hypoxia and glucose deprivation. Hypoxia can be induced by simply replacing oxygen with nitrogen in the incubator, or by adding mitochondrial toxins such as cyanide (chemical hypoxia; Fig. 1). Because oxidative stress and overactivation of glutamate receptors play important roles in ischemic neuronal injury, stroke researchers have performed cell culture studies aimed at understanding how oxidative insults (e.g., exposure to $Fe^{2+}$, hydrogen peroxide, or peroxynitrite) and glutamate endanger neurons. There are many advantages of such cell culture models including: the ability to directly manipulate and monitor, in a highly controlled manner, the environment of the neurons; the ability to directly observe the cell death process; the capability of monitoring various parameters of oxidative stress and ion homeostasis in living neurons; and the ability to establish cause-effect relationships. Of course, any findings obtained in such models ultimately require extension to in vivo animal models and ultimately to human stroke patients.

Morphological changes consistent with apoptosis, particularly cell shrinkage and nuclear DNA fragmentation, occur in cultured rodent brain neurons following exposure to hypoxia (Bossenmeyer et al. 1998), glucose deprivation (Cheng and Mattson 1991; Kalda et al. 1998), glutamate (Ankarcrona et al. 1995), and oxidative insults (Kruman et al. 1997). Measurements of mitochondrial transmembrane potential (Mattson et al. 1993b, 1995; White and Reynolds 1996; Bruce-Keller et al. 1999a), and membrane permeability transition and release of cytochrome *c* (Hortelano et al. 1997) have documented mitochondrial alterations known to occur in cells undergoing apoptosis in several of the cell culture models. Cyclosporin A, an inhibitor of the mitochondrial permeability transition, protected cultured neurons against apoptosis induced by ischemia-relevant insults (Keller et al. 1998), and protected hippocampal neurons against hypoglycemic injury in vivo (Friberg et al. 1998). Activation of caspase-3 occurs in cultured hippocampal and cortical neurons following exposure to excitotoxic and metabolic insults (Mattson et al. 1998; Tenetti et al. 1998). Caspase inhibitors protect cultured neurons against metabolic and excitotoxic insults (Mattson et al. 1998), demonstrating a key role for caspase activation in the cell death process.

Proteins that may play key roles in executing the neuronal death process following ischemic injury are being identified in studies of cell culture models. The proapoptotic protein Par-4 is induced in cultured hippocampal neurons following exposure to glutamate (Duan et al. 1999a) and oxidative insults (Chan et al. 1999). Studies using antisense technology and expression of a dominant-negative form of Par-4 showed that Par-4 is a key link in the chain of events leading to mitochondrial dysfunction, caspase activation, and nuclear apoptosis (Guo et al. 1998; Chan et al. 1999; Duan et al. 1999a). Hypoxia and/or reoxygenation in cultured forebrain neurons induces c-Jun, Jun B, Jun D, c-Fos, JNK1, and JNK3 in association with delayed neuronal apoptosis (Chihab et al. 1998). Expression and/or activation of these proteins suggests a role for the transcription factor AP-1 in neuronal apoptosis, although its precise role remains to be established.

## Antiapoptotic signal transduction pathways that limit ischemic neuronal injury

A combination of in vivo and cell culture studies have provided convincing evidence that cerebral ischemia results in the activation of several different inter- and intracellular signaling cascades, which serve the function of protecting neurons against the insult (see Mattson and Furukawa 1996; Mattson and Lindvall 1997 for reviews). Neurotrophic factors were the first class of signaling molecules that was shown to protect neurons against insults relevant to the pathogenesis of stroke (see Mattson and Barger 1995 for review). Seminal cell culture studies showed that basic fibroblast growth factor (bFGF) can protect cultured neurons against glutamate toxicity (Mattson et al. 1989). Further work showed that bFGF and several other neurotrophic factors, including NGF, brain-derived neurotrophic factor (BDNF), insulin-like growth factors (IGFs), and platelet-derived growth factor (PDGF) protect cultured hippocampal and cortical neurons against injury induced by glucose deprivation and hypoxia (Cheng and Mattson 1991, 1992, 1994, 1995; Mattson et al. 1993b). Cytokines entered the arena of neuroprotective factors relevant to stroke when it was discovered that TNF-$\alpha$ can protect cultured hippocampal neurons against excitotoxic, metabolic, and oxidative insults (Cheng et al. 1994; Barger et al. 1995). Additional neuroprotective factors initially identified in cell culture studies include transforming growth factor-$\beta$ (TGF$\beta$) and the secreted form of amyloid precursor protein (sAPP$\alpha$; Mattson et al. 1993a; Prehn et al. 1993).

Studies of animal models of stroke support the hypothesis that the brain responds to ischemic insults by activating antiapoptotic signaling pathways that involve neurotrophic factors and cytokines. The expression of several different neurotrophic factors and cytokines is rapidly increased following ischemia-related insults in the rodent brain (see Mattson and Lindvall 1997 for review). For example, levels of bFGF, TNF$\alpha$, and BDNF are increased in cerebral cortex and hippocampus following transient global forebrain ischemia (Endoh et al.

1994; Uno et al. 1997) and focal ischemia reperfusion (Speliotes et al. 1996; Botchkina et al. 1997). Intravenous administration of bFGF reduces infarct volume following focal cerebral ischemia in rats (Fisher et al. 1995) and cats (Bethel et al. 1997). Intraventricular administration of NGF (Shigeno et al. 1991), sAPPα (Smith-Swintosky et al. 1994), and TGFβ (Henrich-Noack et al. 1996) reduced damage to CA1 hippocampal neurons following transient global forebrain ischemia. NGF expression, under control of a c-*fos* promoter, reduced damage following permanent occlusion of the middle cerebral artery in rats (Guegan et al. 1998). Postreperfusion administration of BDNF via infusion into the lateral ventricle protected CA1 neurons against delayed death in the four-vessel occlusion rat model of transient global forebrain ischemia (Kiprianova et al. 1999). BDNF also suppressed activation of astrocytes and microglia as indicated by a decrease in levels of inducible nitric oxide synthase production. TGFβ reduced infarct size in a mouse focal ischemia model in which the middle cerebral artery was permanently occluded (Prehn et al. 1993). Direct injection of glial cell-derived neurotrophic factor (GDNF) into the dorsal hippocampus protected CA1 neurons against ischemic injury (Miyazaki et al. 1999).

Studies in which specific neurotrophic factor and cytokine signaling pathways are blocked, through genetic or pharmacological manipulations, have provided insight into the roles of these injury-related factors in the pathogenesis of stroke. For example, data obtained from studies of TNF receptor knockout mice have shown that ischemia-induced TNF serves a neuroprotective function following focal ischemia reperfusion (Bruce et al. 1996; Gary et al. 1998). Administration of the drug clenbuterol to adult rats increased levels of NGF in cerebral cortex and reduced infarct volume following permanent focal ischemia; administration of NGF antisense oligonucleotides abolished the protective effect of clenbuterol, indicating a key role for endogenous NGF in the neuroprotective mechanism (Culmsee et al. 1999).

Cell culture studies have played a critical role in identifying the mechanisms whereby neurotrophic factors and cytokines protect neurons against ischemic injury (see Mattson and Furukawa 1996 for review). In the case of neurotrophins (NGF, BDNF, NT-3, and NT-4/5), bFGF and IGFs the ligands activate receptors with intrinsic tyrosine kinase activity. The activated receptors then dimerize and transautophosphorylate each other which, in turn, activates a cascade of kinases such as mitogen-activated protein (MAP) kinases that ultimately lead to transcription factor activation. Although the gene targets that play key roles in antiapoptotic actions of neurotrophic factors are still being elucidated, recent studies of cultured embryonic rat hippocampal neurons suggest that they include: antioxidant enzymes such as SOD and glutathione peroxidase (Cheng and Mattson 1995; Mattson et al. 1995, 1997a, 1997b); calcium-binding proteins such as calbindin (Cheng et al. 1994); and excitatory amino acid receptors (Mattson et al. 1993c; Cheng et al. 1995).

Cytokines activate distinct but overlapping neuroprotective signaling pathways. For example, TNF activates receptors linked to the cytoplasmic transcription factor NF-κB (Fig. 6; and see Mattson et al. 2000b for review). NF-κB consists of two subunits, p50 and p65, which comprise the transcription factor dimer, and I-κB, which is an inhibitory subunit. Signals that activate NF-κB induce phosphorylation of I-κB, which causes it to dissociate from the other two subunits, and the latter subunits then translocate to the nucleus, where they act on κB-responsive genes. NF-κB activation mediates the neuroprotective actions of TNF in culture and in vivo (Barger et al. 1995; Mattson et al. 1997b), apparently by inducing the expression of genes encoding Mn-SOD (Bruce et al. 1996; Mattson et al. 1997b; Yu et al. 1999a) and Bcl-2 (Camandola and Mattson 2000; Tamatani et al. 1999). Levels of p65 and NF-κB activity decrease following focal cerebral ischemia, and neurons in the regions where p65 levels decrease and TNF levels increase undergo apoptosis (Botchkina et al. 1999), providing further evidence for an antiapoptotic role for NF-κB. However, although TNF clearly exerts a direct antiapoptotic action on neurons (via activation of the transcription factor NF-κB), it may also activate potentially cytotoxic microglia (see Mattson et al. 2000b for review). The neuroprotective signaling pathway activated by sAPPα involves activation of a receptor linked to elevation of cGMP levels and stimulation of cGMP-dependent protein kinase; this pathway may protect cells via both a transcription-independent pathway involving dephosphorylation (activation) of potassium channels (Furukawa et al. 1996) and a transcription-dependent pathway involving NF-κB (Barger and Mattson 1996). The overall conclusion from studies of mechanisms of action of neurotrophic factors and cytokines is that they activate signaling pathways that lead to suppression of oxyradical production and stabilization of cellular calcium homeostasis.

Antiapoptotic signaling mechanisms activated within cells (i.e., independent of intercellular signals) have recently been discovered and characterized. For example, a cytoskeleton-based antiapoptotic signaling pathway activated by calcium influx was recently described (Furukawa et al. 1995, 1997a, 1997b). Calcium activates a protein called gelsolin, which then cleaves actin filaments. Actin depolymerization, in turn, results in reduced calcium influx through NMDA receptors and voltage-dependent calcium channels (Furukawa et al. 1997b). Apparently actin filaments interact with the channels, probably in an indirect manner involving yet-to-be-determined actin-binding proteins. Studies of gelsolin knockout mice have provided evidence that this actin-based signaling pathway serves a neuroprotective role following excitotoxic and ischemic insults (Furukawa et al. 1997b; Endres et al. 1999). A second example is that NF-κB is activated by oxidative stress and increases in intracellular calcium levels, thereby acting as a "stress sensor" (Mattson et al. 2000b).

A final example is the induction of expression of "heat-shock" proteins. Such proteins are best known for

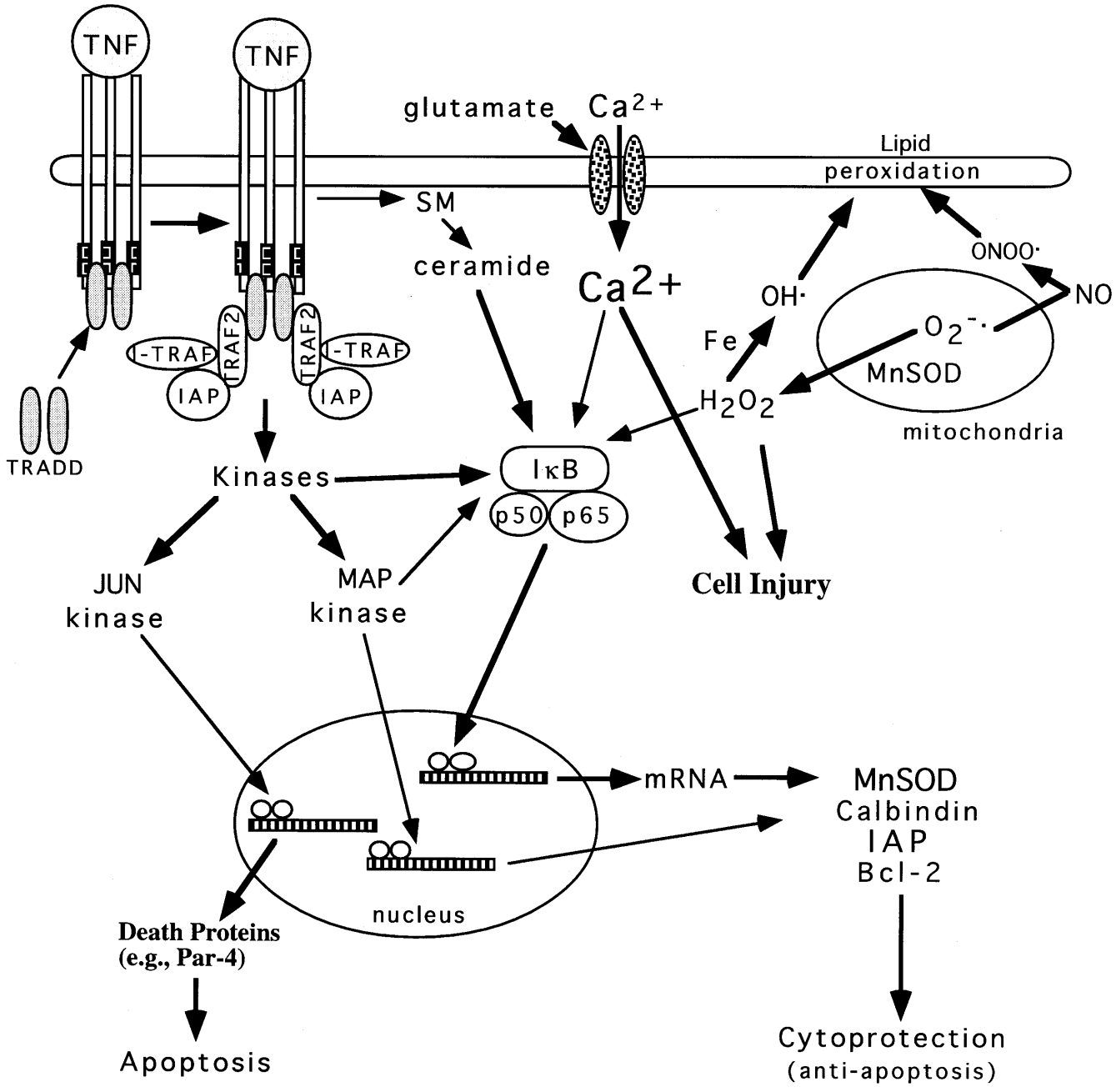


**Fig. 6** Mechanisms whereby activation of nuclear factor (NF-κB) protects neurons against ischemic injury. The prototypical NF-κB complex consists of p65 and p50 (transcription factor dimers) and I-κBα (inhibitory subunit). NF-κB is activated by both inter- and intracellular signals induced by ischemic brain injury, including tumor necrosis factor (*TNF*) which, upon binding its receptor, recruits and activates a series of signaling proteins including TNF receptor-associated death domain (*TRADD*), TNF receptor-associated protein 2 (*TRAF2*) and inhibitor of apoptosis protein (*IAP*). A kinase cascade is thus activated which results in phosphorylation of I-κB. Increases in levels of intracellular calcium and reactive oxygen species (e.g., $H_2O_2$), resulting from ischemia, for example, are important inducers of NF-κB. Antiapoptotic genes induced by NF-κB include those encoding mitochondrial superoxide dismutases (*MnSOD*), calbindin, inhibitors of apoptosis (*IAP*), and some *Bcl-2* family members. Ischemia may simultaneously activate "death" signaling pathways including the JUN kinase pathway (*SM* sphingomyelinase)

their function as chaperones that serve to control protein folding and trafficking, and removal of damaged proteins. Levels of several such chaperone proteins, including HSP-70 and GRP-78, are increased in neurons following cerebral ischemia (Wang et al. 1993; Wagstaff et al. 1996). Studies of cultured neurons have shown that HSP-70 and GRP-78 play pivotal roles in determining whether or not a neuron survives exposure to excitotoxic, oxidative, and metabolic insults (Lowenstein et al. 1991; Yu et al. 1999a; Yu and Mattson 1999). Mice overexpressing human HSP-70 exhibit reduced neuronal damage following permanent focal ischemia compared with wild-type mice, suggesting a key role for HSP-70 in preserving neurons following stroke (Plumier et al. 1997). Interestingly, recent data suggest that levels of

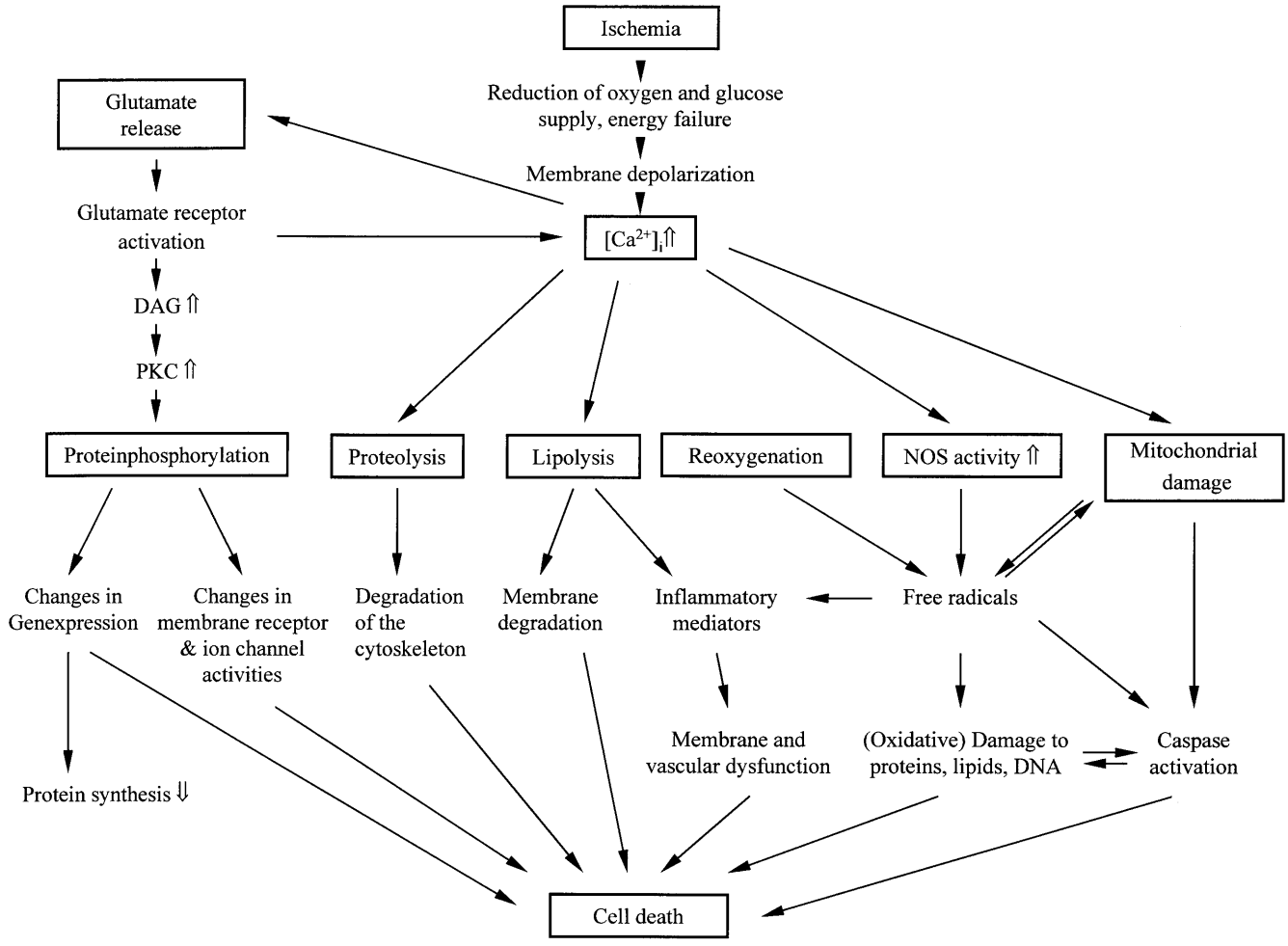


**Fig. 7** Overview of biochemical cascades that occur in neurons in response to cerebral ischemia (*DAG* diacylglycerol, *PKC* protein kinase C, *NOS* nitric oxide synthase). For details on data supporting this scenario, see the text and recent review articles by Dirnagl et al. (1999) and Chan and Mattson (1999)

stress proteins and neuronal resistance to ischemic injury can be influenced by diet. Maintenance of adult rats and mice on a calorie-restricted diet increases resistance of neurons to ischemia and related excitotoxic and oxidative insults, and improves behavioral outcome (Bruce-Keller et al. 1999b; Duan and Mattson 1999; Yu and Mattson 1999). The latter studies provided evidence that the mechanism whereby dietary restriction benefits neurons involves upregulation of stress proteins.

## Conclusion

Cell culture and animal models of ischemic brain injury have proven invaluable in elucidating the cellular and molecular underpinnings of neuronal cell death in stroke. A working model of the cascade of events that leads to neuronal apoptosis following stroke is shown in Fig. 7. Oxyradical production, perturbed calcium homeostasis, caspase activation, and mitochondrial dysfunction appear to be important mediators of neuronal apoptosis following stroke. Evolving technologies for monitoring and manipulating reactive oxygen species, intracellular calcium levels, mitochondrial function and caspase activity provide the opportunity to establish cause-effect relationships in many different paradigms of apoptosis relevant to the pathogenesis of stroke. A variety of signaling pathways that prevent neuronal apoptosis are being identified in cell culture studies. Such pathways often involve induction of genes that encode proteins that have antioxidant functions or that modulate cellular calcium homeostasis. Such studies are identifying compounds and dietary manipulations with antiapoptotic actions that may prove beneficial in preventing neuronal death and improving outcome following stroke in humans.

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