

CHAPTER

35

Brain Ischemia and Reperfusion: Cellular and Molecular Mechanisms in Stroke Injury

Ludmila Belayev, Youming Lu, Nicolas G. Bazan

OUTLINE

Brain Responses to Ischemia	621	<i>Reactive oxygen species enhance the excitotoxic and apoptotic consequences of ischemic brain damage</i>	631
Focal cerebral ischemia	622		
Global cerebral ischemia	623		
Injury in the Ischemic Phase	627	Breakdown of the Neurovascular Unit and Brain Edema	631
Excitotoxic glutamate neurotransmitter	627	<i>Metalloproteinases during the neurovascular unit disruption</i>	631
Excitotoxicity	627	<i>Significance of aquaporins in brain edema</i>	632
Ca ²⁺ overloading in the ischemic injury	627		
NMDA receptors, brain function and cell death	628	Neuroprotection Signaling and Resolution	632
Downstream cell death signals of NMDA receptors	629	<i>of Inflammation: Mechanisms</i>	632
	629	<i>Inflammatory mediators and anti-inflammatory regulation</i>	632
Brain Injury During the Reperfusion Phase:	629	<i>Apoptotic signaling</i>	633
Free Radicals in Ischemia–Reperfusion Injury	629	<i>Docosanoids and penumbra protection</i>	635
<i>Reactive oxygen species contribute to the injury</i>	629		
<i>Mitochondria, nitric oxide synthase and polyunsaturated fatty acid metabolism are sources of reactive oxygen species during ischemia–reperfusion</i>	630	Potential Therapeutic Strategies for Acute Ischemic Stroke	638
<i>Polyunsaturated fatty acids generate reactive oxygen species</i>	630		
<i>Brain antioxidants contribute to the protection of brain from ischemia–reperfusion injury</i>	630	Box: The Stroke Penumbra Is a Translational Target	639
	630		
	630	Acknowledgments	640
	630	References	640

BRAIN RESPONSES TO ISCHEMIA

According to the World Health Organization, 15 million people worldwide have a stroke each year, 6 million of whom will die. Stroke is the second leading cause of death for people above the age of 60 and is the leading cause of long-term disability irrespective of age, gender, ethnicity or country. Despite progress made in understanding the pathophysiology of stroke, today the only efficacious treatment approved for ischemic stroke is thrombolysis. Unfortunately, only a small percent of patients can be elected to undergo this treatment.

Therefore, the need for developing an effective treatment for stroke remains vital. In the United States, stroke continues to be the third leading cause of death, affecting over half a million new victims each year (Lloyd-Jones et al., 2009). Of these, nearly one-third will die and another third will be left with severe and permanent disability. Unlike ischemic injury to other tissues, the severity of disability is not predicted well by the amount of brain tissue lost. For example, damage to a small area in the medial temporal lobe may lead to severe disability, such as loss of speech, while damage to a greater volume elsewhere may have minor consequences on function.

The degree of disability does not simply reflect the severity or distribution of impaired blood supply. Populations of cells lying side by side in the brain can display dramatically different vulnerabilities to equivalent degrees of ischemia. Although a great deal has been learned about ischemia-reperfusion, much remains to be learned about what cellular and molecular mechanisms contribute to the vulnerability of the brain to stroke.

Focal cerebral ischemia

Brain ischemic injury can result from several different processes. Focal ischemia, which accounts for a majority of strokes, occurs when an artery supplying a region of the brain is occluded by an embolus (which often derives from a plaque in an artery or a thrombus from the heart), a thrombus, or a platelet plug that forms on the inner surface of an artery, such as the common carotid artery (Fig. 35-1). While focal ischemic insults reflect the distribution of the vascular supply to a region, the area of infarction is typically less than the entire distribution of the occluded artery because of the presence of collateral circulation at the borders of the region supplied by the occluded vessel (Fig. 35-1). The resulting area of infarction depends on the duration and degree of the vascular occlusion and the magnitude of collateral blood supply (Hossmann,

2009). The region of the brain irrigated by the occluded artery, termed the *ischemic core*, develops severe injury, while the area surrounding the core, termed the *penumbra* (which maintains some blood flow supplied by collateral circulation), sustains less severe injury (Fig. 35-2) (Ramos-Cabrer et al., 2011). Irreversible damage progresses over time from the center of the most severe flow reduction to the periphery, which has less-disturbed perfusion (Fig. 35-2). This progression of irreversible damage is characterized by a complex cascade of electrophysiological, molecular, metabolic and perfusion disturbances. Waves of depolarizations, the peri-infarct spreading depressions, the inducing of ion pump activation, and enhanced release of glutamate all negatively impinge on the drastically increased metabolic demand during reduced oxygen supply. In turn, increasing hypoxic tissue changes and lactic acidosis further contribute to brain damage (Heiss, 2010).

The ischemic penumbra is an area with (1) reduced cerebral blood flow but with relatively preserved high-energy metabolism; (2) zero electrical activity, preserved ion homeostasis and transmembrane potentials; (3) impaired protein synthesis and relatively preserved ATP levels; (4) abnormal perfusion on magnetic resonance imaging (MRI), but normal appearance on diffusion imaging; and (5) an increased oxygen extraction fraction and decreased cerebral blood flow on positron emission tomography (Fisher, 2006). Thus, ischemia in

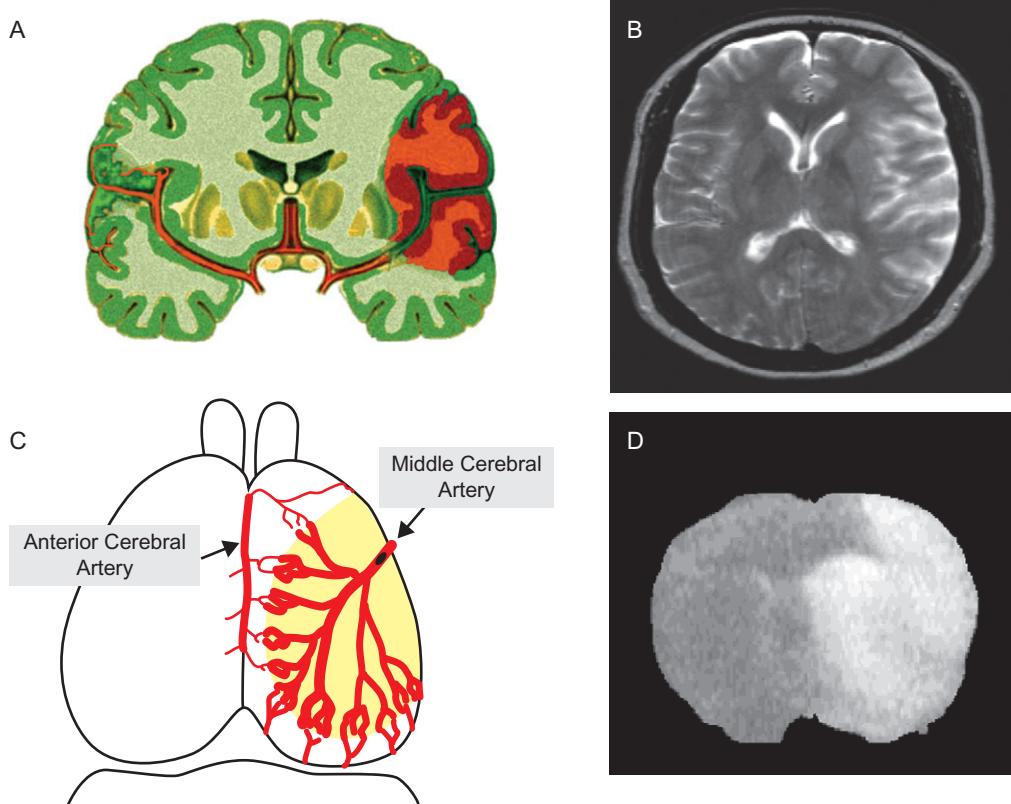


FIGURE 35-1 Panel A: Brain diagram showing the disrupted blood supply in the human brain, caused by a blocked blood vessel, leading to development of brain infarction (depicted in red color). Panel B: Magnetic resonance imaging (T2-weighted image) showing hyperintense lesion and edema (bright regions) 18 hours after stroke. (Courtesy of Karen A. Tong, M.D., Loma Linda University). Panel C: A diagram showing the disrupted blood supply, caused by a blockage of the MCA in the rat's brain, leading to brain infarction (depicted in the yellow color). Panel D: T2-weighted image at 24 h showing a large cortical and subcortical infarction after 2 h of MCA occlusion in rat.

the penumbra causes dysfunctions, but not severely enough to result in irreversible damage. Prompt restoration of perfusion in the penumbra by injection of thrombolytic agents may prevent the onset of irreversible damage in this area, thus limiting neurological deficit (Moskowitz et al., 2010).

The goal for treating ischemic stroke is to salvage as much of the penumbra as early as possible. It has been reported that roughly half of all acute ischemic patients still have intact penumbra on MRI (Rivers et al., 2006), and as such these areas are potentially salvageable. However, only 8% of all ischemic stroke patients are eligible for treatment with recombinant tissue plasminogen activator (r-tPA) (Kleindorfer et al., 2004). Imaging techniques have been used to identify penumbra in patients; such techniques include positron emission tomography (PET), single proton-emission computed tomography (SPECT), MRI, and computed tomography (CT). More recently, the hypoxia indicator ¹⁸F-fluoromisonidazole (FMISO) and the GABA_A receptor ligand ¹¹C-flumazenil (FMZ) have been used to refine the capability of PET to identify the penumbra. The use of ¹⁸F-FMISO PET with quantitative three-dimensional mapping of the penumbra in acute ischemic stroke patients, grouped by time from stroke onset, described a central core to peripheral evolution of infarction at the expense of the penumbra (Ebinger et al., 2009). Areas of increased FMISO uptake were shown in acute stroke patients up to 48 hours after clinical onset (Markus et al., 2004) (Fig. 35-3). CT perfusion (CTP) is helpful in predicting the fate of ischemic tissue. The passage of the contrast agent through the brain can be recorded, and parametric maps of cerebral blood volume (CBV) and cerebral blood flow (CBF), as well as contrast mean transit time (MTT), can be generated (Fig. 35-4).

Another approach for imaging the ischemic penumbra employs central-type benzodiazepine receptor (cBZR) imaging. cBZR is a postsynaptic neuronal receptor that is distributed profusely in the cerebral cortex alone and not in the cerebellar cortex (Oku et al., 2010). The expression of cBZR on the neuronal membrane is a sensitive marker of neuronal

integrity, and therefore cBZR ligand could be used to identify early, irreversible neuronal injury (Fig. 35-5). In addition, there may be hemodynamic improvements with a delay of five days that are not correlated with clinical improvement and mismatches between perfusion and metabolism (Fig. 35-6). Imaging of the penumbra and local cellular responses, such as hypoxia and neuronal integrity, together with blood flow and metabolism, will help evaluate the effects of novel drugs and interventions for ischemic stroke in suggesting which interventions have potential as markers of the efficacy for future therapeutic regimens.

Global cerebral ischemia

Reversible global ischemia, which can occur during cardiac arrest and resuscitation, reflects a transient loss of blood flow to the entire brain; it generally results in the death of certain selectively vulnerable neuronal populations (Pulsinelli, 1992). After cardiac arrest in rodents, neurons in different brain areas show differences in their vulnerability. The hippocampal pyramidal cells of CA1, pyramidal neocortical neurons (layers 3, 5 and 6), Purkinje cells and striatal neurons have the highest vulnerability. In the hippocampus, the pyramidal neurons in the CA1 area and some neurons in the hilus are most vulnerable, whereas most of the CA3 pyramidal neurons survive; also, the granular cells in the dentate gyrus are resistant to ischemic damage. For example, pyramidal neurons in the CA1 subfield of the hippocampus die after 10–20 min of global cerebral ischemia, while neurons in the nearby CA3 region are preserved (Fig. 35-7). Ischemic cell death becomes manifested first in the hilar neurons, but cell death in the CA1 region occurs after a delay of one to two days. One week after ischemia, complete ablation of the CA1 pyramidal cell layer can be observed. There are also differences between cell types in their vulnerability. Neurons are more sensitive when exposed to ischemia than are glial cells because neurons have higher energy demands and only neurons can produce glutamate.

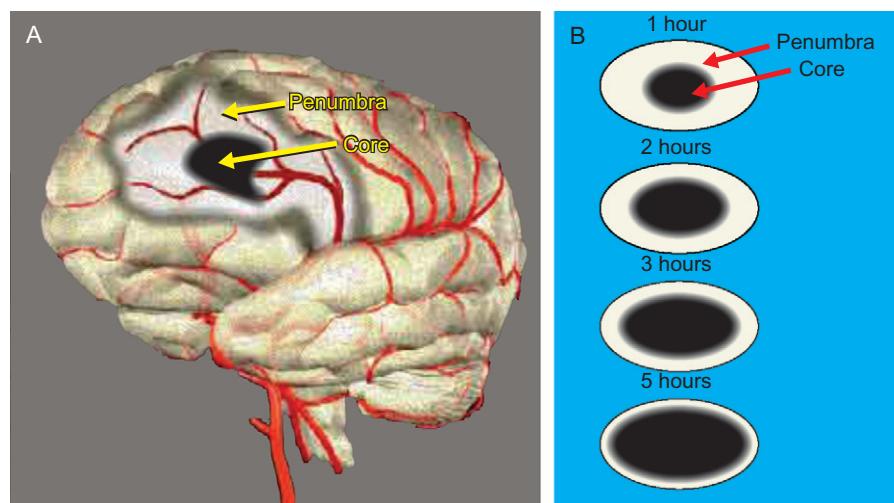


FIGURE 35-2 Panel A: Schematic drawing showing ischemic core and penumbra in the human brain after blockage of the MCA. Panel B: Diagram of evolution of the ischemic penumbra. The initial penumbra amounts to about one-half of the total ischemic lesion. Over the first several hours post-stroke, the ischemic penumbra deteriorates and contributes to enlargement of the ischemic core.

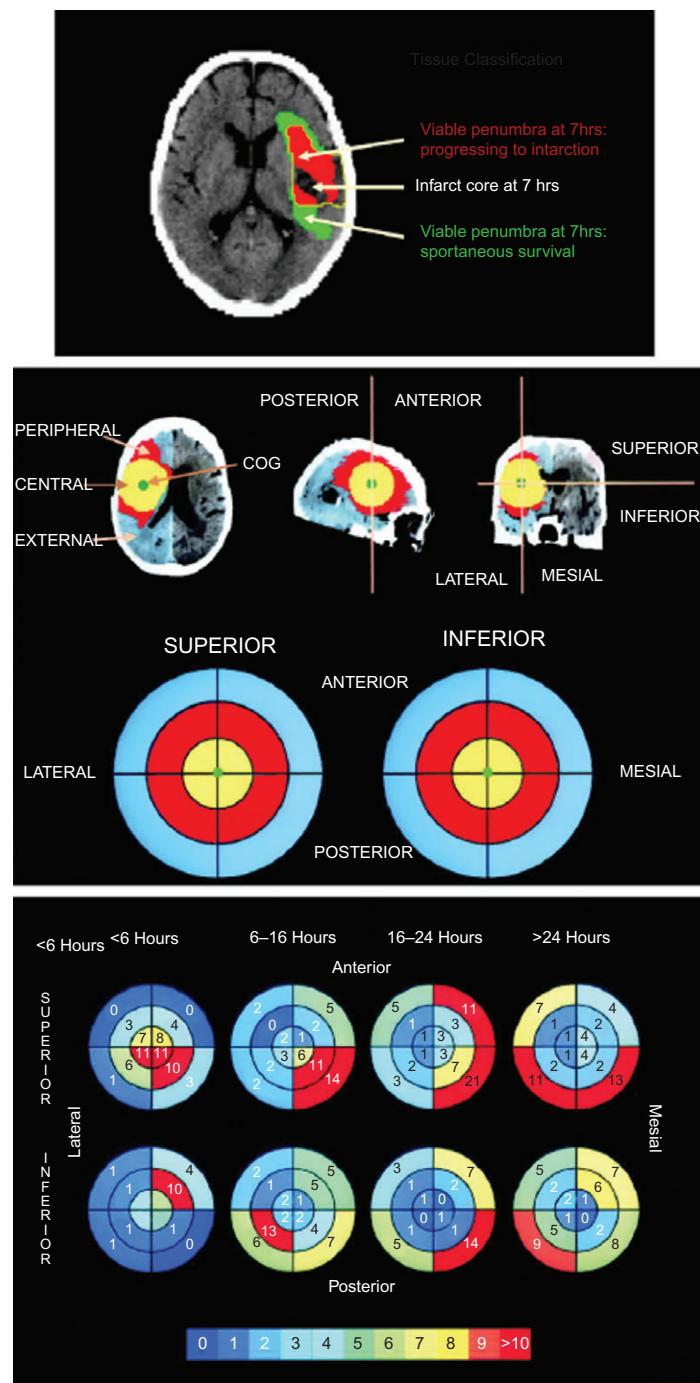


FIGURE 35-3 The stroke penumbra as seen on positron emission tomography (PET) (Markus et al., 2004) **Top:** ^{18}F -fluoromisonidazole (FMISO) PET scan performed 7 hours after stroke onset. The PET scan is co-registered with the delayed CT scan outlining the final infarct at 7 days. At 7 hours after stroke onset, a significant volume of penumbral tissue that is potentially viable is observed surrounding the infarct core. The final infarct volume (outlined in yellow) includes a substantial brain tissue volume that was penumbral and potentially viable 7 hours after stroke onset. **Middle:** Construction of the penumbragram. Central (yellow), peripheral (red), and external (blue) zones of the infarcted volume are shown. In the penumbragram, superior and inferior halves of the infarcted volume are defined by the horizontal plane. The inner, middle, and outer circles represent the central, peripheral, and external zones. Further subdivisions in the anterior/posterior and mesial/lateral planes give 12 regions in each half. Displaying the percentage of voxels with significant FMISO uptake that anatomically correspond to each of these 24 regions generates a map demonstrating three-dimensional distribution of hypoxic tissue. **Lowest:** Temporal evolution of the penumbragram. Composite penumbragram for each time epoch (<6, 6-16, 16-24 and 24-48 hours) after stroke onset are shown. The number in each region refers to the percentage of hypoxic volume. Higher volume of hypoxic tissue is observed in the central region within 6 hours. At later time points, hypoxic tissue is more prevalent in the periphery or externally. Superior, mesial and posterior predominance is seen at all time points. Original image taken from Ebinger et al. (2009) with permission from Elsevier.

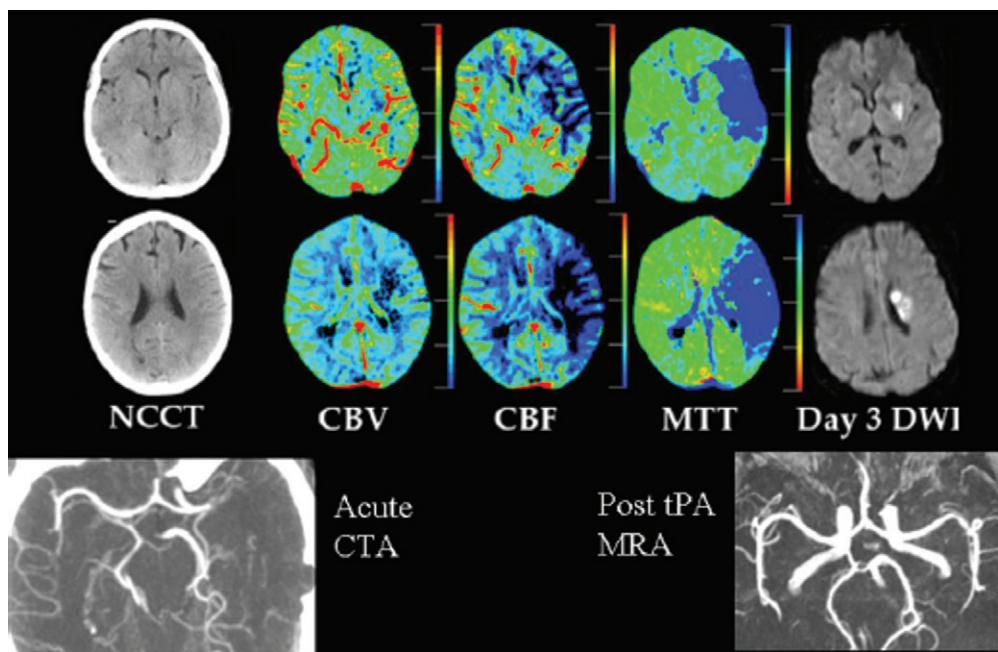


FIGURE 35-4 The penumbra on CT perfusion in a patient imaged with multimodal CT at 3.5 hours after stroke onset with National Institute of Health Stroke Scale (NIHSS) 14. M1 occlusion on CT angiography (CTA) (lower left). Small area of reduced cerebral blood volume (CBV) in lentiform nucleus and deep white matter but surrounded by much larger areas of reduced cerebral blood flow (CBF)/prolonged mean transit time (MTT) consistent with ischemic penumbra. Minimal change seen on non-contrast CT (NCCT). Patient treated with intravenous (IV) thrombolysis and had major early neurologic improvement, complete recanalization on follow-up magnetic resonance angiography (MRA) (lower right), and only a small amount of infarction on follow-up diffusion-weighted imaging (DWI) (corresponding to pre-treatment areas of reduced CBV). Original image taken from Ebinger et al. (2009) with permission from Elsevier.

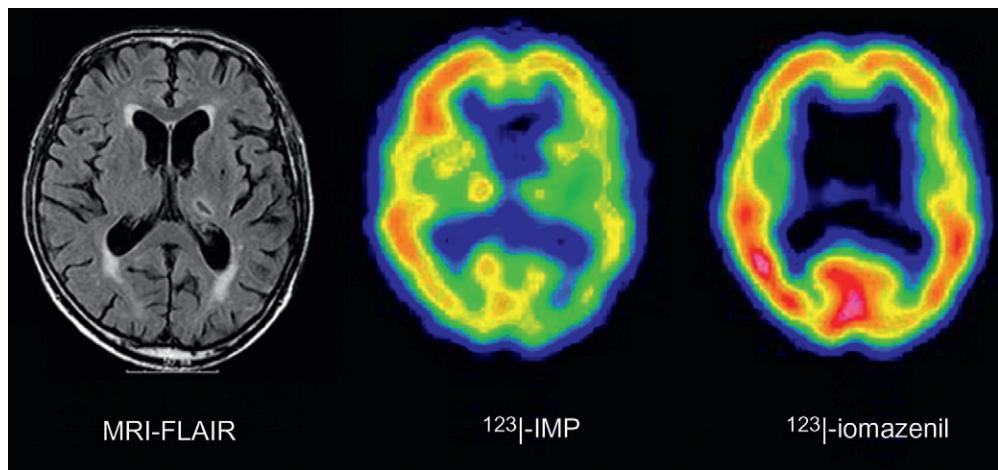


FIGURE 35-5 Fluid-attenuated inversion recovery image of magnetic resonance imaging (MRI-FLAIR) and multitracer SPECT images in a patient with subacute left thalamic lacunar infarction without major artery obstruction. There is no apparent cortical lesion except for mild periventricular hyperintensity on MRI-FLAIR. Perfusion SPECT using ¹²³I-IMP demonstrates mild hypoperfusion in the left hemispheric cortices and the thalamus. Central benzodiazepine receptor SPECT imaging using ¹²³I-iomazenil demonstrates slightly reduced accumulation in the left frontal and temporal cortices and mildly reduced accumulation in the left occipital cortex. This discrepancy between perfusion and ¹²³I-iomazenil accumulation in the left frontal and temporal cortices suggests diaschisis because neuron integrity seems to be preserved except in the left occipital cortex. In the left occipital cortex, there may be neuronal damage for some unknown reason. Original image taken from Oku et al. (2010) with permission from Springer.

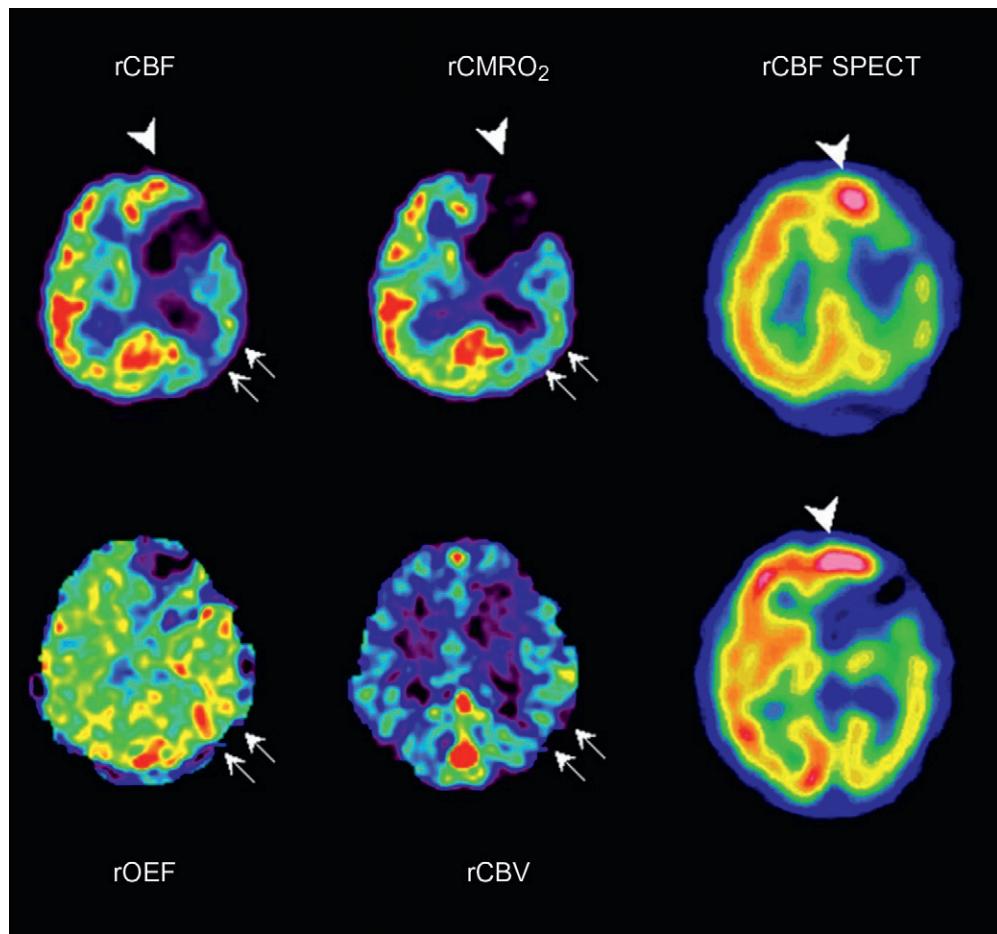


FIGURE 35-6 Subacute left hemispheric stroke due to left internal carotid occlusion. Oxygen-15 gas PET study (left and center columns) clearly demonstrates luxury perfusion in the left frontal cortex (arrowhead). Perfusion SPECT using ^{99m}Tc -HMPAO (right column) demonstrates hyperperfusion and hyperfixation in the same region (arrowhead). Misery perfusion is also seen in the left temporo-occipital region (small arrows). Original image taken from Oku et al. (2010) with permission from Springer.

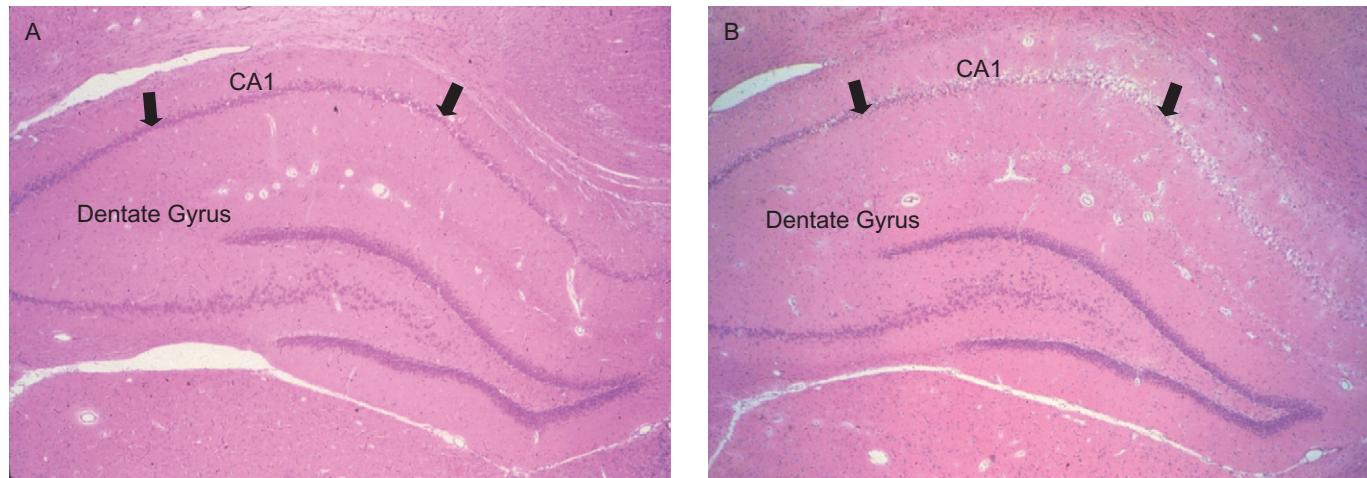


FIGURE 35-7 Rat hippocampus showing neuronal populations selectively vulnerable to ischemic damage. (A) control and (B) ischemia. A brief period of 12 min of global cerebral ischemia causes nearly complete loss of neurons in the CA1 region (arrows) of the hippocampus.

INJURY IN THE ISCHEMIC PHASE

Excitotoxic glutamate neurotransmitter

Glutamate is the most common excitatory neurotransmitter. In small amounts, it is indispensable for neuronal function. In excessive amounts, it is a neuronal poison, a toxin, and has been called excitotoxic. In addition, the brain depends on a second-by-second supply of oxygen and glucose by the blood. A drop in cerebral perfusion can cause a critical shortage of energy. Neurons use up glucose and oxygen faster than they are supplied and discharge glutamate with the same result. Obviously, lack of energy causes initial electrical failure and, if it lasts long enough, results in arrest of cellular functions and cell death.

Excitotoxicity

Normal energy metabolism in the brain has several unusual features, including a high metabolic rate, limited intrinsic energy stores, and critical dependence on aerobic metabolism of glucose. Reflecting this special metabolic status, as well as the existence of several unique injury mechanisms discussed below, the brain exhibits higher vulnerability to ischemic injury than most other tissues. Ischemic brain injury occurs in several clinical settings. When brain hypoxia or ischemia occurs, tissue energy demands cannot be met, so ATP levels fall. Loss of ATP results in decreased function of active ion pumps, such as Na,K-ATPase, the most important transporter for maintaining high intracellular concentrations of K⁺ ($\approx 155 \text{ mmol/l}$) and low intracellular concentrations of Na⁺ ($\approx 12 \text{ mmol/l}$). Loss of ion pump function can result in rundown of transmembrane ion gradients, leading to membrane depolarization, the opening of voltage-sensitive ion channels, and a cascade of subsequent events, which, if sustained, lead ultimately to cell death. Depending on the circumstances, this death may be restricted to selectively vulnerable neuronal populations or may involve all cells in a region of brain, an event termed "brain infarction" (see Membrane Transport, Ch. 3).

Within seconds of an ischemic insult, normal brain electrical activity ceases as a result of the activation of membrane K⁺ channels and widespread neuronal hyperpolarization (Kristian et al., 1997). The hyperpolarization may be due to the opening of K⁺ channels responding to acute changes in local concentrations of ATP, H⁺ or Ca²⁺, or it may reflect altered nonheme metalloprotein association with and regulation of specific K⁺ channels (Haddad et al., 1997). However this response, while presumably protective, fails to preserve high-energy phosphate levels in tissue, since concentrations of phosphocreatine (PCr) and 'ATP fall within minutes after ischemia onset (Welch et al., 1997). The fall in Po₂ during ischemia leads to enhanced lactic acid production as cells undergo a Pasteur shift from a dependence on aerobic metabolism to a dependence on glycolysis. The resulting lactic acidosis decreases the pH of the ischemic tissue from the normal 7.3 to intra-ischemic values ranging from 6.8 to 6.2, depending in part on the pre-ischemic quantities of glucose available

for conversion to lactic acid. In addition, efflux of K⁺ due to the initial opening of K⁺ channels mentioned above results secondarily in prolonged elevations in extracellular [K⁺] and massive persisting neuronal depolarization, a state known as *spreading depression*, which can propagate in brain tissue. Rapid inactivation of O₂-sensitive K⁺ channels by decreased Po₂ may represent one mechanism whereby neurons put a brake on this ongoing K⁺ efflux (Haddad et al., 1997). Other cellular ion gradients are also lost; thus concentrations of intracellular Na⁺ and Ca²⁺ rise while intracellular Mg²⁺ falls. Extracellular concentrations of many neurotransmitters are increased during hypoxia-ischemia. Depolarization-induced entry of Ca²⁺ via voltage-sensitive Ca²⁺ channels stimulates release of vesicular neurotransmitter pools, including the excitatory amino acid neurotransmitter glutamate. At the same time, Na⁺-dependent uptake of certain neurotransmitters, including glutamate, is impaired. High capacity uptake of glutamate by the glutamate transporter couples the uptake of one glutamate and two Na⁺ with the export of one K⁺ and one HCO₃⁻ (or OH⁻) (Ch. 3). When the cellular ion gradients are discharged, the driving force for glutamate uptake is lost. In addition, glutamate uptake by the widely expressed astrocyte high-affinity glutamate transporter GLT-1, or excitatory amino acid transporter-2 (EAAT2), and the neuronal transporter, or EAAT3, can be downregulated by free-radical-mediated oxidation of a redox site on the transporter (Trott et al., 1997). Furthermore, since the transporter is electrogenic, i.e., normally transfers a positive charge inward, membrane depolarization can lead to reversal of the transporter, producing glutamate efflux (Nicholls et al., 1990). Thus, both impaired glutamate uptake and enhanced glutamate release contribute to sustained elevations of extracellular glutamate in the ischemic brain. Microdialysis of ischemic rat brain has detected an increase from the resting extracellular glutamate concentrations of 1–2 μmol/l up to concentrations in the high-micromolar or even low-millimolar range.

Ca²⁺ overloading in the ischemic injury

Glutamate is the major excitatory transmitter in the mammalian central nervous system (CNS) and plays an essential role in neural development, excitatory synaptic transmission, and plasticity (Ch. 17). Immediately following ischemia, however, glutamate accumulates at synapses, resulting in extensive stimulation of its receptors, which can eventually be toxic to neurons (Lipton, 2006; Lo et al., 2003). Glutamate activates three classes of ionophore-linked postsynaptic receptors, namely N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate receptors. NMDA receptor toxicity is dependent on extracellular Ca²⁺, and reflects a large amount of Ca²⁺ influx directly through the receptor-gated ion channels (Lipton, 2006; Lo et al., 2003; Simon et al., 1984). As most AMPA receptor channels have poor Ca²⁺ permeability, injury may result primarily from indirect Ca²⁺ entry through voltage-gated Ca²⁺ channels, Ca²⁺-permeable acid-sensing ion channels (Xiong et al., 2004), and possibly via a cleavage of Na⁺/Ca²⁺ exchangers (Bano et al., 2005). Ca²⁺ overload triggers several downstream lethal reactions, including nitrosative stress (Aarts et al., 2002),

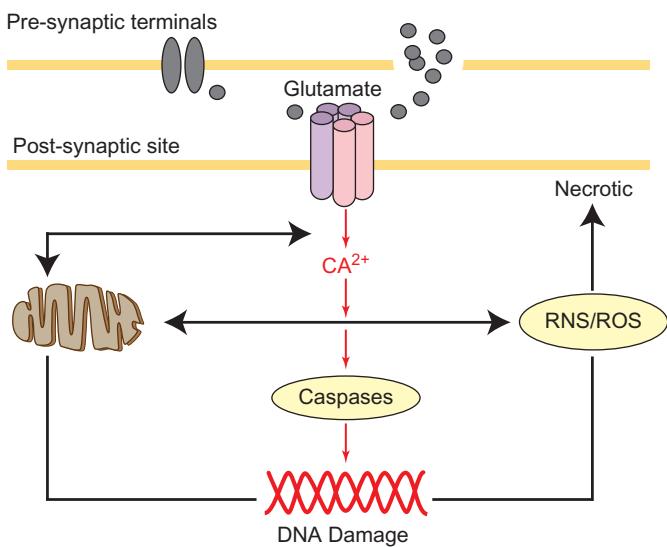


FIGURE 35-8 Ca^{2+} overloading causes neuronal death in ischemia. Ischemic brain accumulates excitatory glutamate transmitter at the synaptic clefts due to increased glutamate release and a decreased glutamate uptake. Glutamate accumulation activates post-synaptic glutamate receptors, leading to the influx of Ca^{2+} into neuronal cells. Ca^{2+} overloading damages mitochondria and hence produces reactive oxygen species (ROS) and nitrogen species (RNS), leading to neuronal necrosis. Ca^{2+} overloading also activates caspases and in turn damages DNA, leading to neuronal apoptosis.

oxidative stress and mitochondrial dysfunction (Fiskum et al., 1999), as illustrated in Fig. 35-8. As noted, overstimulation of glutamate receptors can be considered as a primary intracellular event that induces neuronal death in stroke.

NMDA receptors, brain function and cell death

NMDA receptors are the major subtype of glutamate receptors that participate in rapid excitatory synaptic transmission. Native NMDA receptors appear to be hetero-oligomeric complexes consisting of an essential NR1 subunit and one or more regulatory NR2 subunits (NR2A-D), and the more recently identified NR3 subunit (see also Ch. 17). Since the NR2C- (and possibly NR2D-) containing receptors have very low probability for their channel opening, both NR2A- and NR2B-containing NMDA receptors are considered as the main types of functional NMDA receptor channels in CNS neurons. Activation of NMDA receptors requires two coincident events: (1) binding of the co-agonists glutamate and glycine, and (2) simultaneous membrane depolarization, which removes the Mg^{2+} blockade of the channel pore, leading to the influx of Ca^{2+} . Under physiological conditions, the entrance of Ca^{2+} produces partial inhibition of NMDA receptors via Ca^{2+} -dependent inactivation, thereby preventing the intracellular Ca^{2+} overload (Krupp et al., 1999). Under pathological conditions, however, this negative feedback in Ca^{2+} regulation of NMDA receptors is disabled, resulting in excessive Ca^{2+} influx through the receptor channels (Wang et al., 2003a). Ca^{2+} overload triggers multiple intracellular events that induce irreversible death of neuron cells (Lipton, 2006;

Lo et al., 2003) (see further details of Ca^{2+} dysregulation in Chs. 3, 17, 24, 37).

The riddle of NMDA receptor involvement in both neuronal function and in neuronal death has been partly clarified by the recognition that NMDA receptor-induced responses depend on the receptor location: synaptic NMDA receptors induce neuroprotective, homeostatic signaling, whereas stimulation of extrasynaptic NMDA receptors mediates cell death signaling. These differences result from the activation of distinct gene circuits and from the induction of counteracting signaling pathways for circuitry plasticity and survival. Perturbations in the balance between synaptic and extrasynaptic NMDA receptor activities contribute to neuronal dysfunction in stroke and neurodegenerative diseases. Therefore, neuroprotective, therapeutic approaches should focus on enhancing synaptic activity and downregulating extrasynaptic NMDA receptors.

Calcium ion is a key mediator of neuronal functions that depend on electrical activity. Impairment of calcium homeostasis impinges on amyloid precursor protein (APP) processing, thereby increasing accumulation of β -amyloid ($\text{A}\beta$), which contributes to the pathology of Alzheimer's disease (AD) Ch. 46). One possible consequence of stroke is impairment of APP processing and the onset of AD-like changes. Overactivation of extrasynaptic NMDA receptors, but not synaptic NMD receptors, augments the neuronal production of $\text{A}\beta$. This indicates the existence of distinct signaling pathways coupled to each pool of receptors. Therefore, these data suggest that chronic activation of extrasynaptic NMDA receptors promotes amyloidogenic APP expression leading to neuronal $\text{A}\beta$ release. Clearly there is a connection between the extrasynaptic NMDA receptor and other conditions that lead to ischemia-reperfusion injury, AD and other cognitive impairments.

Although overstimulation of NMDA receptors contributes to stroke damage (Lipton, 2006; Lo et al., 2003), blocking them is deleterious because targeting these receptors also blocks their physiological actions. Thus, an ideal approach for stroke therapy should inhibit the specific NMDA receptor "cell death signals" whereby the pathological effects of NMDA receptors are selectively blocked, leaving the physiological action/s unaffected (Tu et al., 2010). Recently, NR2B subunits of NMDA receptors have become a major focus of stroke research because of their unique features (Tu et al., 2010; Hardingham et al., 2010) First, NMDA receptor NR2B subunits are localized predominantly at extrasynaptic sites. This feature enables the receptor to detect the initial extrasynaptic glutamate spillover in the brain immediately after ischemic insults. Second, stimulation of extrasynaptic NMDA receptors activates specific signals that induce delayed neuronal apoptosis, which occurs a few hours, or even days, after ischemic insults (Hardingham et al., 2010). However, it is now believed that NMDA receptor NR2B subunits also may be combined with NR1/NR2A subunits to form NR1/NR2A/NR2B complex receptors at synaptic sites (Thomas et al., 2006). Thus, using NMDA receptor NR2B subunit antagonists for selectively blocking extrasynaptic receptors still could be problematic. To date, efforts toward stroke therapy using NMDA receptor antagonists have failed. Several explanations have been proposed for this failure (Lo et al., 2003), but the most important

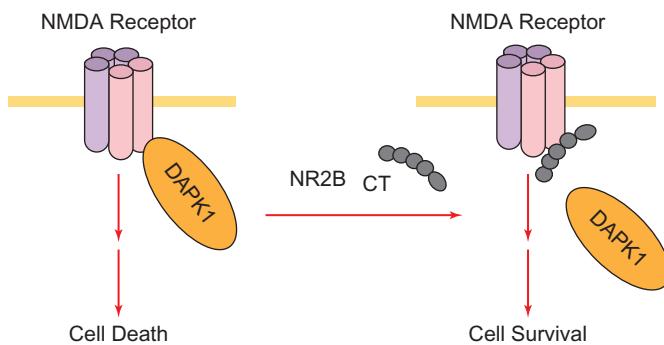


FIGURE 35-9 DAPK is linked with NMDA receptor toxicity in stroke damage. After stroke, the increased extracellular concentration of glutamate causes overflow of synaptic glutamate and excitotoxic activation of extra-synaptic NMDARs. The increase in Ca^{2+} influx through NR2B-containing NMDARs activates DAPK1 and sequentially binds to NMDA receptor NR2B subunit, leading to neuronal cell death. Application of NR2B-derived interference peptide (NR2B_{CT}) inhibits the binding of DAPK1 to NR2B subunit and hence causes neuronal survival.

ones are (1) inhibition of the receptor physiological functions; (2) limited therapeutic time windows; and (3) insufficiency to protect against non-neuronal cell injury in the brain.

Downstream cell death signals of NMDA receptors

Stroke insults lead to an early necrotic cell death by a complex pathological cascade, including energy depletion, oxidative stress, and peri-infarct depolarization. There are also some delayed mechanisms, such as inflammation and apoptosis. The intervention of these delayed cell death events allows the exploration of a longer therapeutic time window, as in the cases of inhibitions of caspase-3 and c-Jun N-terminal kinase (Borsig et al., 2003) (see Ch. 37). More recent studies have focused on NMDA receptor NR2B subunit downstream signaling via interaction with PSD-95 (Aarts et al., 2002). Interfering with NR2B-PSD-95 interaction by uncoupling neuronal nitric oxide synthase (nNOS), including the interference peptide Tat-NR2B9c, protects versus brain infarction in rats with focal cerebral ischemia (Aarts et al., 2002). This finding suggests that blocking NR2B-PSD-95 interaction eliminates NMDA receptor toxicity. However, this strategy has been challenging to implement due to concomitant inhibition of synaptic plasticity. Also, some other studies have indicated that genetic deletion of PSD-95 enhances brain damage in stroke (see also Table 25-4 and NMDAR in Ch. 26).

Recently, DAPK1 (death-associated protein kinase 1) is reported to act as a specific NMDA receptor “cell death signal” at extrasynaptic sites (Tu et al., 2010). DAPK1 is a member of the $\text{Ca}^{2+}/\text{CaM}$ -dependent, Ser/Thr kinase family (see Ch. 25) and functions as a pro-apoptotic enzyme (Van Eldik, 2002); also, it directly binds to the NMDA receptor NR2B C-terminal tail, which consists of amino acid 1297-1304 (NR2B_{CT}). A cell membrane permeable NR2B_{CT} peptide (Tat-NR2B_{CT}) is able to specifically block DAPK1-NR2B interaction both *in vitro* and *in vivo*. When Tat-NR2B_{CT} was intravenously

administered two hours after stroke onset, it protected against brain damage, leading to improved neurological functions (Tu et al., 2010). Thus, DAPK1 interaction with NMDA receptor NR2B subunit is a central mediator in stroke damage that opens the exploration of selective blockage of the DAPK1 signaling cascade(s) downstream of the extrasynaptic NMDA receptor as a potential therapeutic approach for stroke (Fig. 35-9).

BRAIN INJURY DURING THE REPERFUSION PHASE: FREE RADICALS IN ISCHEMIA–REPERFUSION INJURY

Reactive oxygen species contribute to the injury

Evidence for free radical production and oxidative stress during ischemia–reperfusion comes from a variety of studies. While free radicals may be generated to a small extent during ischemia, far greater production of reactive oxygen intermediates occurs after reintroduction of oxygen during reperfusion. Early studies with animal models of ischemia–reperfusion injury showed decreased brain concentrations of antioxidants such as ascorbic acid, vitamin E and glutathione, as well as production of aldehydic lipid peroxidation products, detected as thiobarbituric acid-reactive substances. Additional evidence for generation of reactive oxygen species (ROS) has been obtained with (1) intra-ischemic microdialysis with solutions containing salicylic acid or spin-trapping agents (which react with ROS to form relatively stable products that can be detected in the microdialysis solution), or (2) oxidation-sensitive fluorescent probes, such as dihydroethidine, which detect superoxide radicals. Oxidative damage to macromolecules has provided another index of ROS-mediated injury in ischemia–reperfusion. Lipid- and protein-oxidation products, as well as DNA-oxidation products, have been detected in brain tissue after ischemia–reperfusion.

Treatment with antioxidants such as vitamin E, 21-aminosteroids, and spin-trapping agents such as phenyl tert-butyl N-nitron (PBN) can reduce ischemic brain infarction. In addition, transgenic mice that overexpress the cytosolic antioxidant enzyme CuZn-SOD (SOD1; see below) (Sugawara et al., 2003) or the extracellular superoxide dismutase (EC-SOD) (Sheng et al., 1999) are resistant to ischemic brain injury compared to wild-type controls. Conversely, reduction of cellular antioxidant defenses can potentiate ischemic injury. *SOD1*^{-/-} knockout mice exhibit enhanced susceptibility to ischemic brain injury, even though they have a grossly normal CNS phenotype under unstressed conditions, such as in the absence of ischemia or nerve transection. This suggests that other antioxidant defenses are adequate to handle physiological concentrations of cytosolic superoxide anion. In contrast, mice lacking the mitochondrial antioxidant enzyme Mn²⁺-SOD (SOD2) die soon after birth, and mice that are heterozygous for the *sod2* gene, meaning they exhibit half the SOD2 activity of wild-type mice, have larger areas of infarction after focal ischemia. A large body of literature now links ischemic injury to oxidative stress produced by both increased production of ROS and impaired defenses against ROS (reviewed in Chapter 34).

Mitochondria, nitric oxide synthase and polyunsaturated fatty acid metabolism are sources of reactive oxygen species during ischemia–reperfusion

ROS generation during ischemia–reperfusion may come from several sources, including NOS activity, mitochondrial electron transport, multiple steps in the metabolism of arachidonic acid and, in some species, xanthine oxidase, which is produced by hydrolysis of xanthine dehydrogenase. In addition, the decreased intracellular pH accompanying ischemia may alter the binding of transition metals such as iron, increasing their participation in the Haber–Weiss reaction (Halliwell, 1992). P450 enzymes and NAD(P)H oxidase are two additional potential sources of oxygen radicals whose contribution to enhanced radical production during CNS ischemia has not been systematically explored. Mitochondria were among the earliest sites of cellular ROS production to be identified. Studies on isolated mitochondria have suggested that as much as 3–4% of O₂ utilized by resting mitochondria may be leaked as superoxide and H₂O₂ (see Ch. 43). Mitochondrial production of ROS can be enhanced by increased electron-transport activity, as well as by disturbances in electron transfer down the transport chain. The lipid electron-transport molecule ubiquinone (Q9, Q10) is the main site of free radical leaks from mitochondria via the ubiquinone cycle. Ubiquinone undergoes a two-electron reduction, first to a semiquinone and then to a diol, at mitochondrial complex I (NADH dehydrogenase, NADH:ubiquinone oxidoreductase) or complex II (succinate dehydrogenase) and subsequently delivers the two electrons to the iron–sulfur center of complex III (cytochrome bc₁ in mammalian mitochondria). Inhibition of either complex I or complex III will impair the efficiency of electron transfer and may allow a free semiquinone to be produced. The semiquinone can then interact with O₂ to generate O₂[·] (Fig. 35–6). This mitochondrial ROS leak may be enhanced by elevated intracellular Ca²⁺, exposure to fatty acids or other molecules that alter the physical properties of the mitochondrial membrane, and inhibition of mitochondrial respiratory components. Mitochondria appear to be an important and perhaps dominant source of free radicals in brain tissue subjected to ischemia–reperfusion. Microdialysis studies using salicylate to detect ROS release from ischemic brain cortex show that mitochondrial inhibitors such as rotenone eliminate ROS production during ischemia–reperfusion. Elevated concentrations of intracellular Ca²⁺ and Na⁺, a consequence of energy failure and excitotoxic glutamate receptor stimulation, can be expected to inhibit complex I as well as overproduction of superoxide anion. The resultant oxidative stress may lead to further inhibition of mitochondrial respiratory components, promoting further free radical production in a vicious, feed-forward cycle. Although xanthine oxidase has been implicated in animal models of ischemia, its role in human stroke is less clear (see also in Ch. 36).

Polyunsaturated fatty acids generate reactive oxygen species

Arachidonic acid metabolism is also a source of ROS production (Schreiber et al., 1986). It undergoes an extensive

array of reactions to biologically active lipids, the eicosanoids (see Ch. 36). These reactions may be accompanied by free-radical production. In particular, cyclooxygenase isoforms and 5-lipoxygenase contain heme iron and may generate a low concentration of superoxide anion constitutively. 12-lipoxygenases, which possess nonheme iron, may not release superoxide anion but may induce lipid peroxidation after translocation to membranes.

Brain antioxidants contribute to the protection of brain from ischemia–reperfusion injury

The high metabolic rate of brain cells implies a high baseline ROS production, and brain cells possess high concentrations of both enzymatic and small-molecule antioxidant defenses. SOD1 may represent as much as 1% of total proteins in the brain; it converts O₂[·] to H₂O₂, which is then further metabolized to water and oxygen by catalase and glutathione peroxidase. The SOD1 gene is located on chromosome 21 and codes for a 16–18 kDa subunit that binds one Cu²⁺ and one Zn²⁺; the active enzyme is a homodimer. SOD2 is a homotetramer and the gene is on chromosome 6. An extracellular, glycosylated form, SOD3, has been shown in rodents to overlap in activity with SOD1 (Zou et al., 2009). Several other enzymes unrelated to CuZn-SOD, for example, the *atx1-HAH* gene product, are also capable of acting as SODs. It remains to be determined to what extent SOD3 and these alternative dismutases complement SOD1 and SOD2 as antioxidant systems in humans.

Catalase and glutathione peroxidase provide two important cellular systems for eliminating H₂O₂. Although it may bind NAD(P)H, catalase (a 56 kDa cytosolic hemoprotein homotetramer that can act without a cofactor) functions as a peroxidase to convert H₂O₂ to water. It can be irreversibly inactivated by oxidation and it demonstrates decreased activity after ischemia–reperfusion. Catalase is more abundant in astrocytes than in neurons and in white matter than in gray matter, but it can be induced in neurons by neurotrophins. There is substantially less catalase activity in brain than in other tissues, such as liver.

The human genes have been identified for four members of the glutathione peroxidase (GPx) family of selenoproteins. Classical GPx (GPx1) is a complex of four 23 kDa subunits. Plasma GPx (GPx3), GPx2 and a fourth enzyme, phospholipid hydroperoxide glutathione peroxidase (PHGPx), are monomers with extensive homology to classical GPx1. All four enzymes contain one selenium atom per subunit in the form of a selenocysteine, and all use glutathione (GSH) as a cofactor to convert H₂O₂ to water. PHGPx is unique in its ability to detoxify not only H₂O₂ but also fatty acid and cholesterol lipid hydroperoxides directly. In addition, it has a cytosolic isoform and an isoform with a mitochondrial targeting sequence. Both GPx1 and PHGPx proteins are present in the brain. The components of the glutathione peroxidase system, GPx, GSH, glutathione reductase and NAD(P)H, are present in the mitochondria as well as in the cytoplasm. Several other proteins, including glutathione S-transferase, may contribute minor peroxidase activity.

Small-molecule antioxidants include glutathione, ascorbic acid (vitamin C), vitamin E and a number of dietary

flavonoids. Because humans, in contrast to most other animals, are unable to synthesize vitamin C, this important antioxidant must be supplied entirely from dietary intake. Other proteins, such as thioredoxin and metallothionein, may also contribute to some extent to the cellular antioxidant pool.

Reactive oxygen species enhance the excitotoxic and the apoptotic consequences of ischemic brain damage

In addition to direct effects of oxidative injury during ischemia–reperfusion, ROS may modify ischemic excitotoxicity by downregulating current through NMDA receptors. However, exposure to oxidative stress can be expected to enhance NMDA-receptor-mediated neurotoxicity by depleting intracellular antioxidant defenses. Free radicals also contribute to apoptosis at several points in the apoptotic cascade, serving as initiators, early signals and possibly late effectors of apoptotic neuronal death (Nakka et al., 2008). It may be this interaction of injury mechanisms (including excitotoxicity, ischemic apoptosis, oxidative injury, inflammation and impaired metabolism) that, in part, makes the brain so vulnerable to ischemic damage.

BREAKDOWN OF THE NEUROVASCULAR UNIT AND BRAIN EDEMA

Ischemia–reperfusion injury is characterized by increased microvascular permeability. A conceptual framework, the neurovascular unit, comprises neurons, the microvessels that supply them, and their supporting cells. Cerebral microvessels consist of the endothelium (which forms the blood–brain barrier), the basal lamina matrix, and the end-feet of astrocytes. Microglial cells and pericytes may also participate in the unit. Communication has been shown to occur between neurons and microvessels through astrocytes. Breakdown of the blood–brain barrier (BBB) during cerebral reperfusion leads to the development of vasogenic edema and infarction, all contributing to cerebral reperfusion injury (Weiss et al., 2009; Aktan et al., 1998). This has been supported by several animal and clinical studies (Belayev et al., 1996; Dobbin et al., 1989; Dankbaar et al., 2010). Permeability of the BBB was increased at 15 minutes after reperfusion of the brain, and cerebral edema occurred 15–30 minutes after reperfusion that followed transient global cerebral ischemia in rats (Sage et al., 1984). Acute disruption of the BBB occurs between three and five hours after a two-hour temporary middle cerebral artery occlusion, and a more widespread increase in regional BBB permeability is present at 48 hours (Fig. 35-10). As a result of the breakdown of the neurovascular unit after ischemia, the tissue content of water in the affected area of the brain may increase, leading to swelling or edema of the ischemic region. Some of the increased tissue water is intracellular, reflecting impaired cellular ion homeostasis due to energy failure, as well as the action of glutamate, which triggers excess cation influx in neurons and glia. Some of the increased tissue water is extracellular, coming from leakage of solute and water from the intravascular space because of breakdown of the BBB

(Sandoval et al., 2008). The disruption of the BBB has also been observed in ischemic stroke patients (Kloska et al., 2010).

Damage to microvascular endothelial cells can lead to vasospasm and can promote adhesion of platelets and leukocytes, leading to vessel plugging. Maneuvers that limit the post-ischemic recruitment of inflammatory cells to the ischemic zone and the subsequent inflammatory response can protect the integrity of the BBB and improve post-ischemic cerebral blood flow (Welch et al., 1997). Knockout mice that do not express intercellular adhesion molecule (ICAM)-1, a protein that is important in promoting adhesion of leukocytes to endothelial cells, are less vulnerable to ischemic injury and have better cerebral blood flow than littermate controls after temporary ischemia (Connolly et al., 1996).

Reperfusion injury is an inflammatory process modulated by the complex signaling mechanisms of the immune system. With reperfusion, additional neutrophils, platelets, and other inflammatory cells from the peripheral circulation rapidly accumulate within the brain tissue in response to the signals of chemotactic agents (Hall et al., 2010). There is swelling of endothelial cells and interstitial edema, both of which lead to increased hydrostatic pressures within the microcirculation and narrowed capillary lumina. There is loss of adrenergic control of vascular tone and loss of production of vasodilating agents such as nitric oxide from the endothelial cells. Leukocytes and platelets plug the postcapillary venules, further impairing capillary flow (Mehta et al., 2007).

Agents that protect endothelial cells, such as antioxidants, can help preserve neurovascular unit integrity following ischemia–reperfusion (Hall et al., 1996). The response of nonvascular or parenchymal cells to the ischemic insult also affects BBB function. For example, NMDA receptor antagonists, which reduce neuronal death and tissue infarction after focal ischemia, also decrease BBB disruption, possibly by blocking NMDA-receptor activated neuronal production of ROS, thus reducing their deleterious effects on endothelial or astrocyte cell function (see also neuroinflammation in Ch. 34).

Metalloproteinases during the neurovascular unit disruption

Matrix metalloproteinases (MMPs) also contribute to disruption of the neurovascular unit during ischemia (Ramos-Fernandez et al., 2011; Jin et al., 2010). MMPs are Zn²⁺ endopeptidases that are secreted as the precursor zymogen, and they are then cleaved to the active form of the protein. MMP-2 and MMP-9 are type IV collagenases that have been shown to degrade the perivascular extracellular matrix following ischemia, thereby causing disruption of the neurovascular unit. Inhibition of MMPs limits BBB opening and decreases edema formation following ischemia (Lee et al., 2010). It has been suggested that MMPs have a role in promoting hemorrhagic transformation after stroke, especially in the context of tissue plasminogen activator administration as thrombolytic therapy to stroke patients (Lo et al., 2004).

Cerebral edema plays a central role in the pathophysiology of cerebral ischemia. The formation of cerebral edema results in an increase in tissue water content and brain swelling which, if left unchecked, can lead to elevated intracranial pressure (ICP), reduced cerebral blood flow, progressive

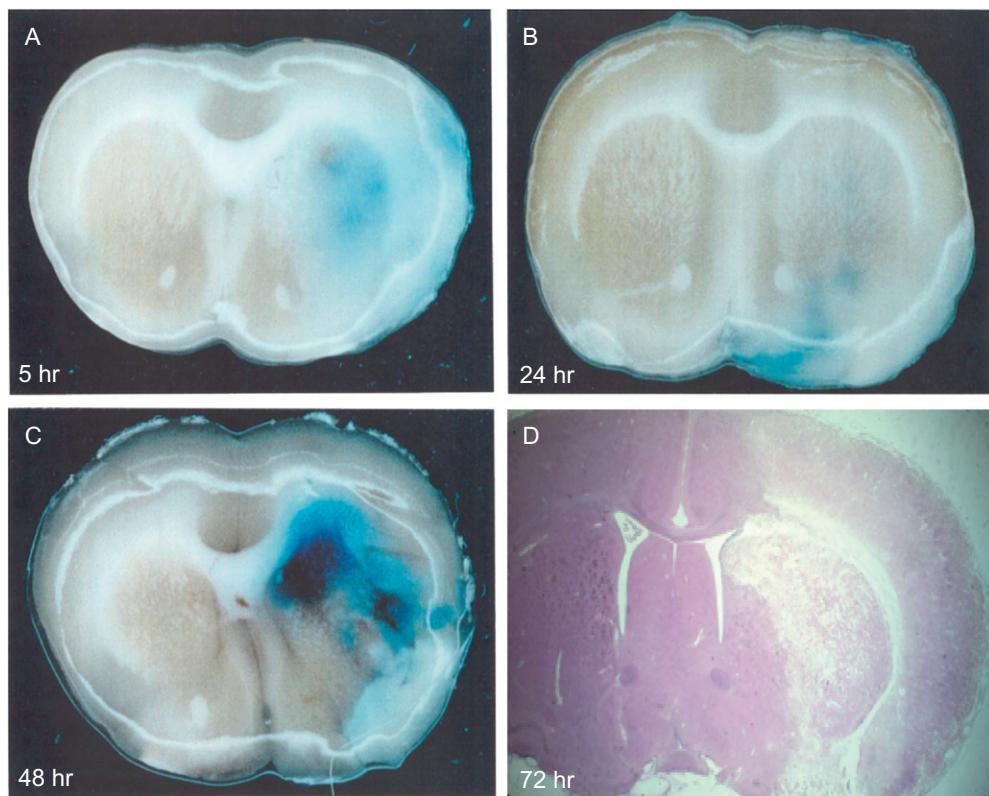


FIGURE 35-10 Neurovascular unit disruption after 2h of temporary middle cerebral artery occlusion in rats. Panels A-C: coronal brain slices, illustrating loci of Evans Blue (EB) extravasation at 5h (panel A); 24h (panel B) and 48h (panel C) after reperfusion. EB extravasation is grossly visible in the 5h and 48h but is less noticeable in the 24h. **Panel D:** paraffin-embedded section stained with hematoxylin and eosin from rat sacrificed at 72h post-MCAo. An extensive infarct involves the dorsolateral and lateral portions of neocortex and the entire striatum.

neurologic deterioration, and ultimately cerebral herniation and death (Simard et al., 2010). Managing patients with malignant cerebral infarction remains one of the foremost challenges in medicine. Despite the clinical significance of cerebral edema, the mechanism of brain water transport and edema formation remains poorly understood. The release of osmotically active substances (arachidonic acid, electrolytes, lactic acid) from necrotic brain tissue causes cerebral edema. This is aggravated by vascular injury and leakage of proteins into the interstitial space. Various cytokines peak in the serum within the first 24 hours of an acute stroke, and are thought to initiate the cascade of tissue damage. At the site of ischemia itself, activated leukocytes release free radicals and toxins causing further destruction. By three to four days, interstitial fluid accumulates in the infarct and around it. This is the most dangerous period for a large cerebral infarction.

Significance of aquaporins in brain edema

Recently, *aquaporins* have been implicated in edema formation after ischemia (Zador et al., 2009). Aquaporins are small, homotetrameric membrane channels that mediate rapid water flux through cell membranes (see Aquaporins in Ch. 3). The main aquaporin in the brain, AQP4, is assembled into multi-monic square arrays (orthogonal arrays) in astrocyte foot process membranes adjacent to capillary basement membranes.

Like AQP1, AQP4 is constitutively active and is therefore believed to be involved in normal regulation of brain water flux. However, AQP4 knockout mice develop less post-ischemic edema and smaller infarcts than do littermate controls, suggesting that AQP4 may contribute to early edema formation during ischemia–reperfusion injury (Agre et al., 2002). Finally, because tissue edema in the rigid intracranial cavity can restrict blood flow, secondary ischemia can ensue.

NEUROPROTECTION SIGNALING AND RESOLUTION OF INFLAMMATION: MECHANISMS

Inflammatory mediators and anti-inflammatory regulation

Inflammation is a cardinal host defense response to cerebral ischemia. Inflammatory mediators have relatively few actions in healthy CNS tissue and are expressed at very low, or undetectable, levels. However, they are induced rapidly in response to cerebral ischemia, in which case they exert diverse actions. Some elements that may be involved in this inflammatory response include macrophages, endothelial cells, neutrophils, lymphocytes and platelets, along with non-cellular elements, such as the complement system, the blood

coagulation cascade, reactive oxygen species, nitric oxide, and pro- and anti-inflammatory cytokines in addition to other mediators. Under these conditions, microvascular perfusion also might be impaired (Lucas et al., 2006).

The presence of damaged cells and debris causes ramified resting microglia to transform into rounded migratory macrophages (de Groot & Rauen 2007). In this active state, microglia produce cytokines and trophic factors that can exert damaging or protective effects on neighboring cells. Cytokines can also cross the BBB probably either by active transport or through leaky regions of endothelia when the BBB is compromised by a pathological condition. Thus, the CNS can be affected not only by inflammatory mediators produced within the brain, but also through the actions of mediators originating from the periphery. Proinflammatory cytokines and other mediators play an essential role in CNS inflammation through the induction of chemokines and adhesion molecules, recruitment of immune cells into the parenchyma, and activation of immune cells and endogenous glial cells (Rothwell et al., 2000).

Reactive oxygen species may be generated by activated macrophages, neutrophils, endothelial cells, and platelets (Allen et al., 2009). Mediators such as cytokines and chemokines may be formed by macrophages, lymphocytes, neutrophils and endothelial cells. Decreased/increased nitric oxide (NO) formation, increased formation of endothelin-1 and blood coagulation may result in disturbances of microvascular perfusion. Several of the compounds released during the inflammatory response, such as reactive oxygen species, the cytokine TNF- α , and high concentrations of NO, may produce cytotoxicity and thus induce additional cell injury. Cell injury occurring during the reperfusion period additionally perpetuates the inflammatory response.

The accumulation of leukocytes, along with red blood cells and platelets during reperfusion, may plug capillaries, preventing the restoration of blood flow and resulting in a "no-reflow" phenomenon and secondary cerebral ischemia (del Zoppo, 2006; Pan et al., 2007). Animal studies provide evidence of the deleterious effects caused by leukocytes (Zhang et al., 1994). Neutrophil accumulation was observed at the site of neuronal injury six hours after two hours of middle cerebral artery occlusion (MCAO) in rats. In addition, the infarct volume increased dramatically between 6 and 24 hours following the start of reperfusion, and the period of maximal infarct expansion correlated closely with the time course of neutrophil infiltration (Zhang et al., 1994). The mechanisms of leukocyte infiltration are characterized by leukocyte activation and leukocyte–endothelial interaction, resulting in accumulation in the vascular bed, followed by leukocyte extravasation into the interstitial space. There was significant capillary obstruction caused by leukocytes reperfusing the microvascular bed within 60 minutes after the restoration of cerebral (del Zoppo et al., 1991). Once the leukocytes infiltrate the parenchyma, they then release various chemical mediators including neutrophil elastase, ROS, leukotrienes and prostaglandins, resulting in increased microvascular permeability, edema, thrombosis and parenchymal cell death (Lucas et al., 2006; White et al., 2000).

In response to a brain insult, astrocytes become activated, increasing expression of glial fibrillary acidic protein, and producing cytokines; they also contribute to the formation of the

glial scar, which isolates the damaged area but also acts as a barrier to reinnervation (Lucas et al., 2006). Prevention of this reactive astrogliosis might be predicted to be beneficial for repair and recovery, but in fact prevention increases neuronal loss from the injury site and inhibits repair of the neurovascular unit and axonal remyelination (Faulkner et al., 2004), partly because astrocytes produce neurotrophic factors such as nerve growth factor and brain-derived growth factor that are upregulated by injury. Similarly, microglia produce proinflammatory mediators, but they also have beneficial effects, such as removing debris and harmful compounds from the brain parenchyma (although their phagocytic capacity is limited).

During the first week after stroke, there is a transient inflammatory reaction, especially around blood vessels and in the meninges, due to release of arachidonic and other fatty acids (Ringelstein et al., 2005). As the core of the infarct disintegrates, endothelial cells from the periphery proliferate and capillaries grow into the dead tissue. Neovascularization (which accounts for contrast enhancement) peaks at two weeks. Monocytes from the blood stream enter the infarct through damaged vessels. They ingest the products of degradation of neurons and myelin and are transformed into lipid-laden macrophages. Macrophage reaction appears early and peaks at three to four weeks. Astrocytes from the surrounding undamaged brain proliferate and form a glial scar around the infarct. This is completed in approximately two months. After that, the infarct remains unchanged. With maturation of new capillaries and glial scar formation, the blood–brain barrier is once again sealed. Adult infarcted neurons ordinarily do not regenerate, so some brain tissue is lost forever. Discussion of the biological regulation of axonal regeneration is found in Chapter 32.

Apoptotic signaling

Ischemia–reperfusion–injury–associated inflammatory reactions that occur at the blood–endothelium interface are extremely critical to the pathogenesis of tissue damage. There is evidence to suggest that besides necrosis, apoptosis does contribute significantly to the cell death subsequent to ischemia–reperfusion injury. The major pathways involved in apoptotic signaling may be classified into extrinsic and intrinsic. A schematic view of apoptotic cascades employed by both extrinsic and intrinsic pathways is shown in Fig. 35-11. Apoptosis is discussed in detail in Chapter 37.

One factor that may promote apoptosis after ischemic insults is deprivation of growth factor support (see Growth Factors in Ch. 29). Deprivation may result from damage to neuronal or glial targets responsible for providing growth factor support. However, tissue concentrations of several growth factors increase in the brain following hypoxic–ischemic insults, suggesting that either there may be decreased sensitivity of neurons to neurotrophins after ischemia, or that increased concentrations of neurotrophins are required to counter proapoptotic stimuli, such as free-radical exposure. Addition of exogenous growth factors, such as nerve growth factor (NGF) or basic fibroblast growth factor (bFGF), can reduce hypoxic–ischemic damage. However, while neurotrophins may attenuate ischemic apoptosis, they may, in

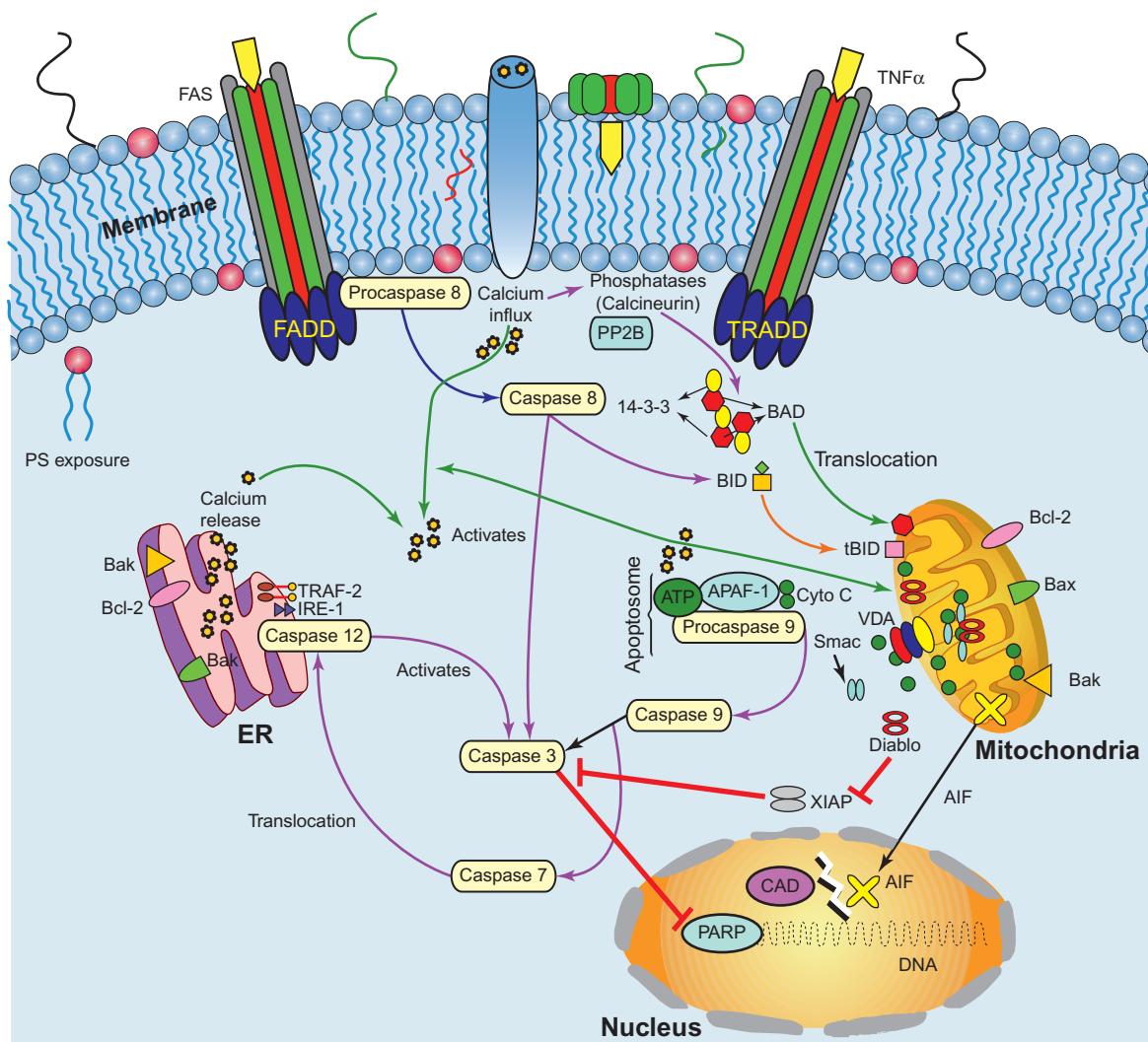


FIGURE 35-11 Apoptotic signal transduction pathways (extrinsic and intrinsic). In the extrinsic pathways, death receptor (e.g., Fas) activation fosters formation of multiprotein DISC that includes the receptor adaptor (e.g., FADD). DISC is the site of activation for procaspase-8, where it attains its active form by oligomerization. Caspase-8 can directly activate caspase-3, an effector caspase; on the other hand, it also cleaves proapoptotic BID into tBID, which translocates to mitochondria to execute apoptosis. In the intrinsic pathway, excessive influx of calcium into intracellular space causes disruption of normal homeostasis, which affects the function of subcellular organelles such as mitochondria and ER. Altered activity of protein phosphatase (e.g., calcineurin) causes translocation of BAD to promote mitochondrial apoptotic pathway. The release of cytochrome c from mitochondrial intermembrane space results in caspase-3 activation via apoptosome complex. The activity of caspases is negatively regulated by IAPs, XIAP, etc., whereas Smac/DIABLO neutralizes their effect. The AIF is released from mitochondria and is translocated to the nucleus. ER undergoes stress upon depletion of calcium from its lumen. ER stress induces activation of caspase-12, which is specifically localized to the ER membrane and may be essential for ER stress-induced apoptosis with further activation of caspase-9 and caspase-3. Both the caspase-7 and tumor necrosis factor receptor-associated factor-2 (TRAF-2) activate caspase-12 in vitro. Moreover, efflux of calcium from ER might trigger secondary activation of mitochondria. Anti- and pro-apoptotic Bcl-2 family members are localized to both the mitochondria and the ER. Caspases cleave key structural components of the cytoskeleton and nucleus. The CAD is responsible for internucleosomal cleavage of DNA, which is a characteristic feature of apoptosis. Original image taken from (Nakka et al., 2008) with permission from Humana Press.

contrast, have deleterious effects by enhancing the excitotoxic necrosis induced by ischemia. The mitogen-activated protein kinases (MAPKs) phosphorylate specific serine and threonine residues of target protein substrates and regulate a variety of cellular activities ranging from gene expression, mitosis, movement, metabolism, and programmed death. Mammalian cells encode three major subfamilies of MAP kinases that include ERK, JNK or stress-activated protein kinases (SAPK),

and p38 MAP kinases (Ch. 25). Upon stimulation, these three kinases relay signaling pathways that lead to various cellular responses (Fig. 35-12).

Persistent impairment of cellular energy metabolism after an ischemic insult may also play a role in triggering apoptotic neuronal degeneration. Studies in cell culture and animal models of stroke suggest that inhibition of mitochondrial function by mitochondrial toxins such as 3-nitropropionic

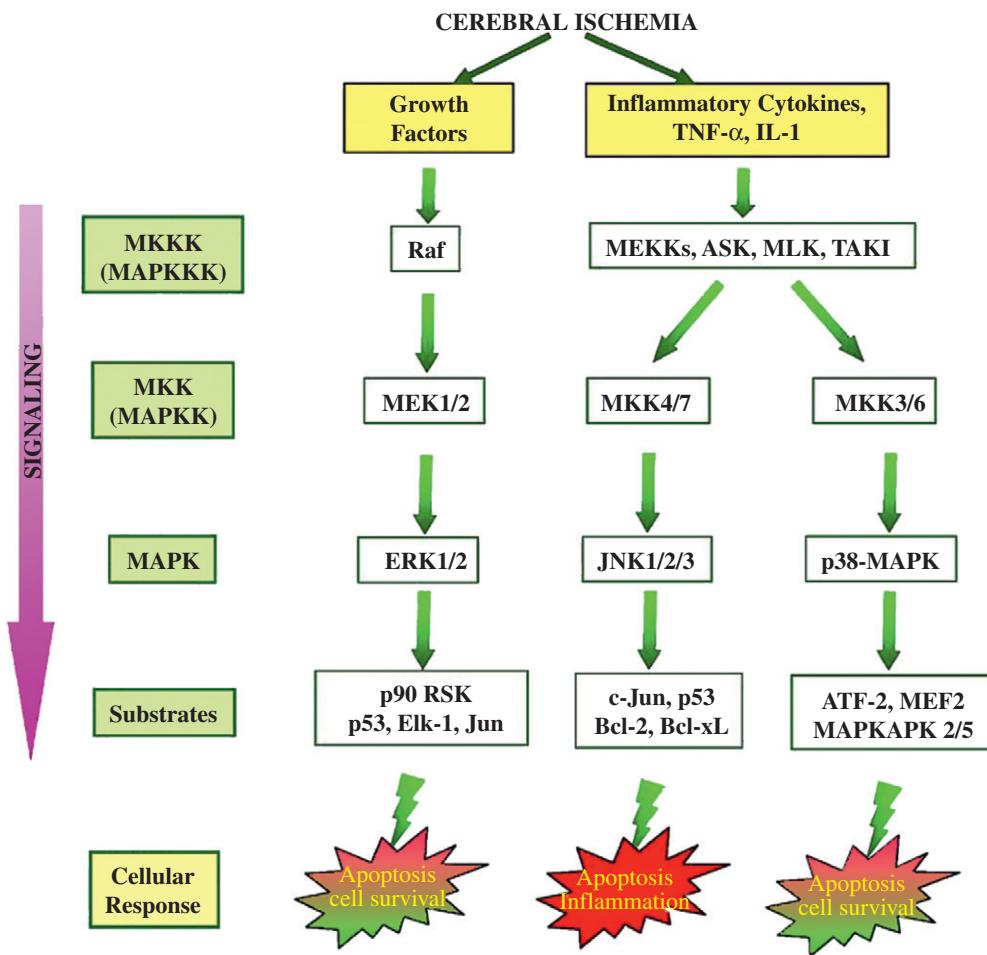


FIGURE 35-12 T MAPKs (ERKs, JNKs and p38) in relay of signaling pathways upon stimuli, the three tier regulatory cascade within each module (MAPKKK, MAPKK and MAPK levels) and the various cellular responses elicited by MAPK control. Original image taken from Nakka et al. (2008), with permission.

acid or rotenone not only worsens excitotoxic injury but also can trigger apoptotic neuronal death. Prolonged deficits in mitochondrial function and energy metabolism have been observed after ischemia–reperfusion, and these deficits may represent triggers for apoptotic neurodegeneration after ischemia. Increased expression and enhanced concentrations of inflammatory cytokines, such as interleukin (IL)-1 β , tumor necrosis factor (TNF) α and transforming growth factor (TGF) β , are observed in brain following ischemia. These factors may derive from inflammatory cells, such as macrophages or microglia, as well as from neurons and glia. Because cytokines are capable of triggering apoptosis in many cell types, including neurons, increased concentrations of these molecules might be expected to trigger apoptotic cell death in vulnerable cells. Thus, ischemic apoptosis may be induced by free radicals, cytokines, metabolic insults and changes in growth factor sensitivity, all of which may result from excitotoxic damage to intracellular systems such as the cytoskeleton, axonal transport, and mitochondria. Moreover, apoptotic cross-talk between major subcellular organelles suggests that therapeutic strategies should be optimally directed at multiple targets/mechanisms for better therapeutic outcome.

Docosanoids and penumbra protection

Cerebral ischemia triggers the rapid accumulation of free fatty acids, mainly arachidonic acid (AA; 20:4 n-6) and docosahexaenoic acid (DHA; 22:6, n-3), due to increases in intracellular calcium and activation of phospholipases (see Ch. 36). Arachidonic and docosahexaenoic acids are then converted via enzymatic processes to eicosanoids or docosanoids, respectively. In addition, these polyunsaturated fatty acids are subjected to free radical-mediated lipid peroxidation. The resulting products are endowed with pro- and anti-inflammatory bioactivities.

DHA belongs to the essential omega-3 fatty acid family and is involved in brain and retinal development, aging, memory formation, synaptic membrane function, photoreceptor biogenesis and function, and vision (Lukiw et al., 2005; Bazan, 2009). DHA is found in cold-water fatty fish, including salmon, tuna, mackerel, sardines, shellfish, and herring. DHA has potent anti-inflammatory properties. Since inflammation is at the root of ischemia–reperfusion and of many chronic diseases, DHA treatment has shown beneficial effects in patients with coronary heart disease, asthma, rheumatoid

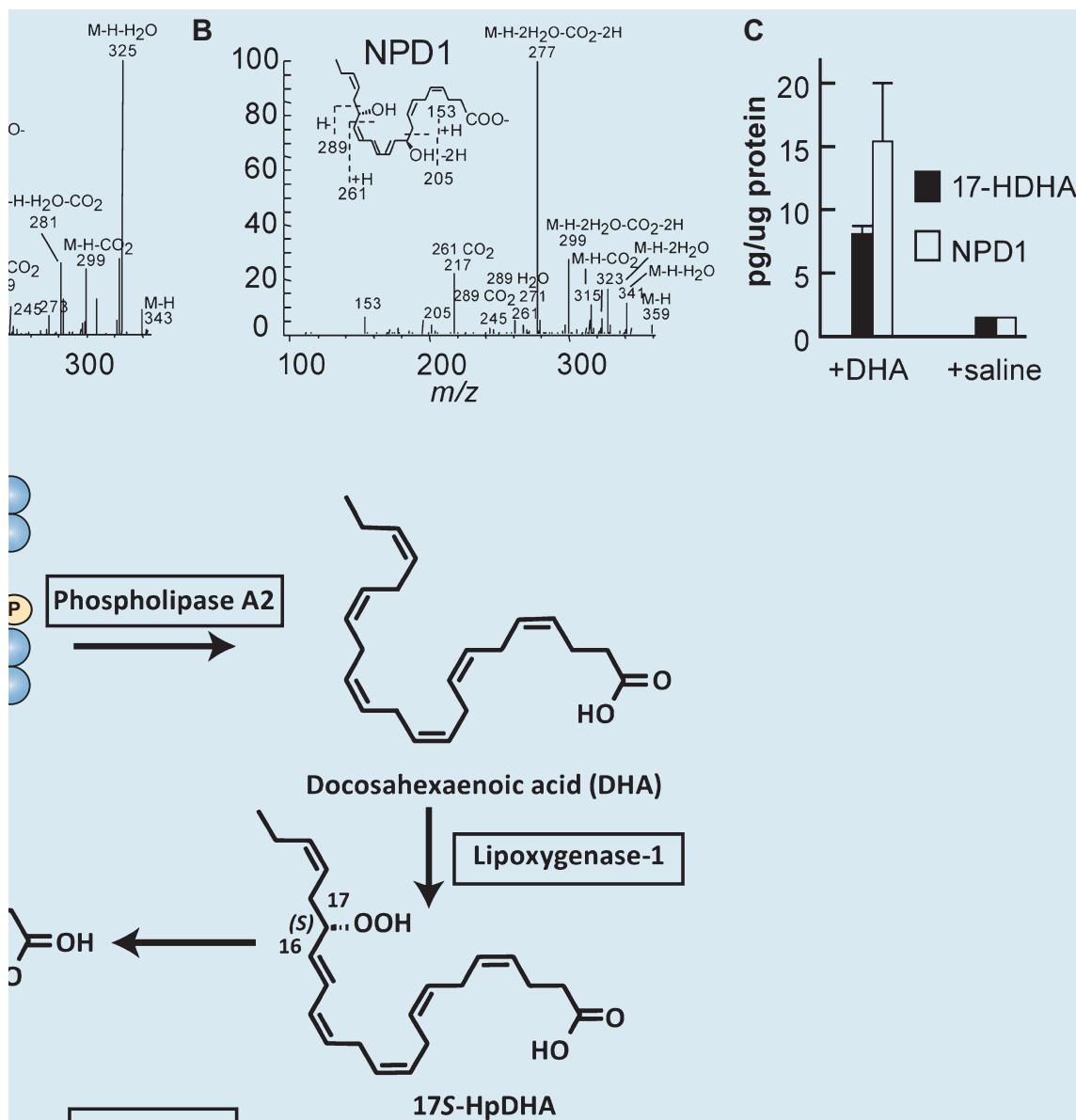


FIGURE 35-13 The characterization and quantification of 17-HDHA and NPD1 in the ipsilateral penumbra 3 days after MCAo. (A–B) The fragmentation pattern is depicted and (C) the quantification is presented. The increased content of 17-HDHA and of NPD1 in the ipsilateral penumbra in animals injected with DHA is consistent with the activation of the biosynthesis of NPD1. (D) Enzyme-mediated oxygenation of DHA for the biosynthesis of NPD1. Phospholipase A2 releases DHA from the second C position of phospholipids during brain ischemia-reperfusion. 15-Lipoxygenase-1 catalyzes the synthesis of 17S-H(p)DHA, which in turn is converted to a 16(17)-epoxide and then is enzymatically hydrolyzed to NPD1. Original image taken from Belayev et al. (2011) with permission.

arthritis, osteoporosis, sepsis, cancer, dry eye disease, and age-related macular degeneration (Simopoulos, 2008). Moreover DHA is protective in ischemia (Bazan, 2009) and in spinal cord injury (Ward et al., 2010).

The recent identification of the docosanoid neuroprotectin D1 (NPD1; 10R,17S-dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid) in the brain ischemia–reperfusion paradigm (Marcheselli et al., 2003), as well as in neural cells exposed to oxidative stress (Bazan, 2005), has uncovered a key survival-signaling event leading to neuroprotection. DHA is precursor to the stereospecific mediator neuroprotection D1 (NPD1).

The synthesis from DHA is through lipoxygenation by 15-lipoxygenase-1 (15-LOX-1), then epoxidation and finally, hydrolysis to NPD1 (Fig. 35-13). NPD1, which is a pleiotropic modulator of inflammation resolution (Serhan et al., 2008), acts against apoptosis, promotes cell survival, inhibits brain ischemia–reperfusion–mediated leukocyte infiltration and pro-inflammatory gene expression, promotes neurogenesis, attenuates edema formation, and reduces stroke volume 48 h after MCAO onset (Marcheselli et al., 2003; Rodriguez de Turco et al., 2002; Belayev et al., 2005). The administration of human serum albumin complexed with DHA results in

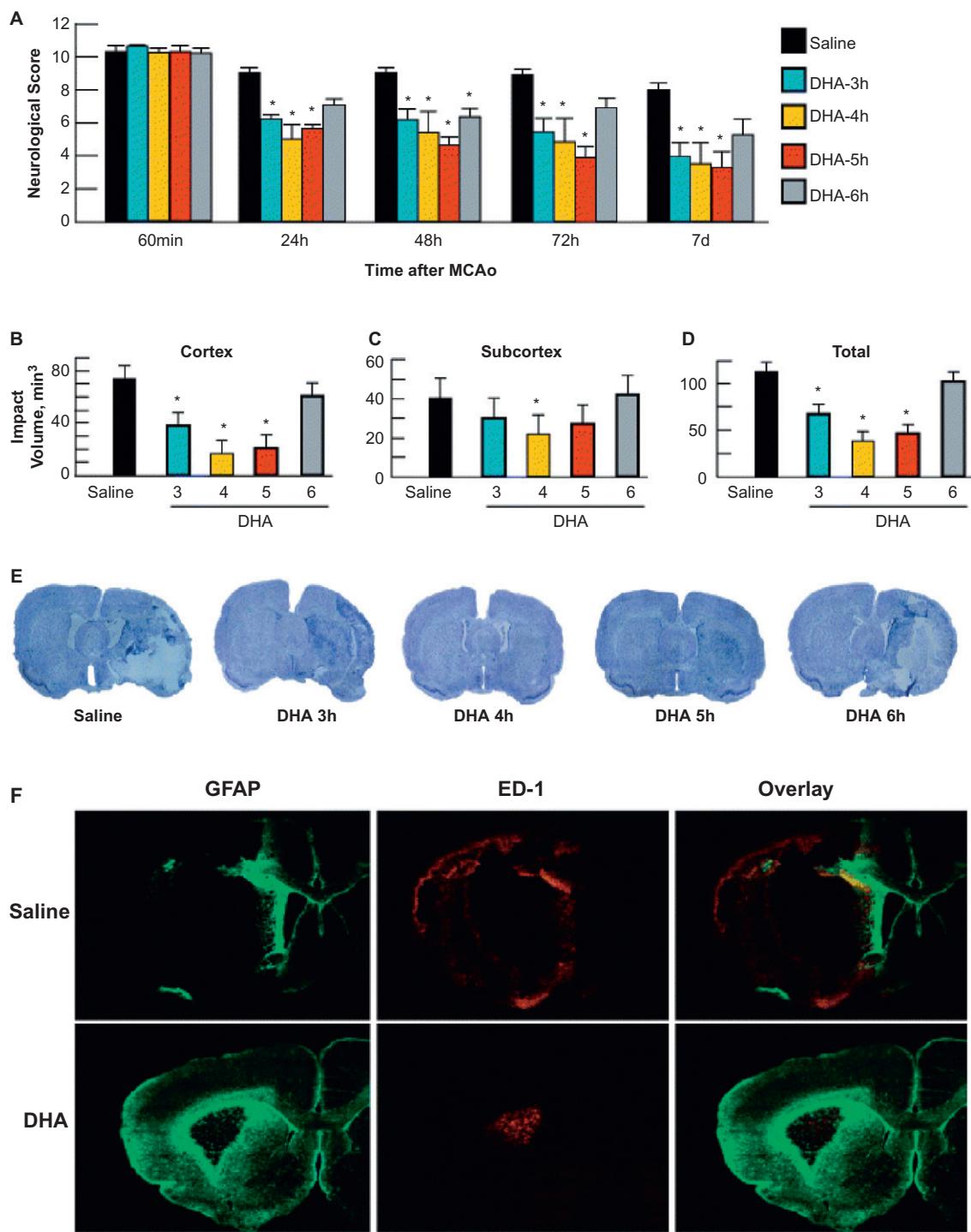


FIGURE 35-14 Therapeutic window potential of DHA: (A) Neurological score (normal score = 0; maximum score = 12) was improved after DHA administration when administered 3, 4, and 5h after onset of stroke. Cortical (B), subcortical (C), and total corrected infarct volumes (D) on day 7. DHA reduced cortical and total infarct volumes when administered 3, 4, and 5h after stroke. Data are means \pm SEM. Asterisk, significantly different from saline ($p < 0.05$; repeated-measures ANOVA followed by Bonferroni tests). (E) Computer-generated MosaiX-processed images of Nissl-stained paraffin-embedded brain sections from rats treated with saline or DHA at 3, 4, 5, and 6h after the onset of ischemia. Saline-treated rat shows large cortical and subcortical infarction. In contrast, rats treated with DHA at 3, 4, and 5h show less extensive damage, mostly in the subcortical area. DHA-treated rat at 6h shows infarct involving cortical and subcortical regions. (F) Computer-generated MosaiX-processed images of GFAP (green), ED-1 (red), and GFAP/ED-1 double staining (overlay) on day 7 after 2h of MCAo at a magnification $\times 10$. Treatment with DHA or saline was given at 3h after onset of stroke. Original image taken from Belayev et al. (2011) with permission.

increased NPD1 production in the ipsilateral hemisphere, but not the contralateral hemisphere, concomitantly with neuroprotection. These results indicate that NPD1 synthesis requires available DHA to then counteract cellular damage during ischemia–reperfusion damage (Belayev et al., 2005).

DHA therapy in low and medium doses improves neurological and histological outcome following focal cerebral ischemia in rats (Belayev et al., 2009). Moreover, DHA improves behavior and reduces total infarct volume by a mean of 40% when administered at 3 h, by 66% at 4 h, and by 59% at 5 h (Fig. 35-14) (Belayev et al., 2011). Total lesion volumes computed from T2-weighted images were reduced in the DHA group at all time points. DHA administration after an ischemic stroke is able to salvage the penumbra and lipidomic analysis showed that NPD1 synthesis in the penumbra three days after MCAO is upregulated under these conditions.

POTENTIAL THERAPEUTIC STRATEGIES FOR ACUTE ISCHEMIC STROKE

Ischemic stroke is an emergency. In the acute phase of ischemic stroke, treatment is focused on saving as much penumbral tissue as possible. The window of opportunity for salvaging the penumbra is very short (Fig. 35-2). If adequate blood supply is not restored within 3 h, necrosis extends to the penumbra (Moskowitz et al., 2010). The restoration of the blood supply can reduce the extent of brain tissue injured by salvaging the portion of reversibly damaged penumbra. One therapeutic modality that has shown efficacy in humans is thrombolysis with either intravenous or intra-arterial infusion of tissue plasminogen activator. This mechanism provides a rationale for clinical trials that have demonstrated that reperfusion after thrombolysis improves clinical outcome in selected patients with acute stroke (Ramos-Cabrera et al., 2011). Reperfusion, however, carries certain risks. Some patients experience disastrous outcomes in the form of fatal edema or intracranial hemorrhage following thrombolysis (Molina, 2011). In some animal stroke models (Yamashita et al., 2010; Zhang et al., 2006), reperfusion after a long ischemic period can cause a larger infarct than that associated with permanent vessel occlusion. Thus, while reperfusion may reduce infarct size and improve clinical outcome in some patients, in others it may exacerbate the brain injury and produce a so-called “cerebral reperfusion injury” (Molina, 2011). Cerebral reperfusion injury can be defined as a deterioration of ischemic but salvageable brain tissue after reperfusion. Thrombolysis and embolectomy (Smith, 2006) usually result in reperfusion of the infarcted brain tissue and therefore carry the risk of causing reperfusion injury. Thus reperfusion injury deserves the attention of those interested in the diagnosis and treatment of acute stroke. Strategies to reduce or minimize cerebral reperfusion injury require understanding the underlying pathophysiology.

With the progress made in the understanding of the mechanisms in cerebral ischemia and reperfusion injury, an increasing number of strategies have been developed for limiting or preventing further brain damage during reperfusion (Moskowitz et al., 2010). Unimodal targeting of key events in stroke pathophysiology is not effective in providing long-term benefits, thusly leading to negative results in previous

clinical neuroprotective stroke trials. Because of the complex, netlike intra- and extracellular processes of ischemia–reperfusion injury and multiple injury pathways, it is hard to achieve effective neuroprotective treatment by a single compound. Strategies targeting these processes include multiple combination therapies as well as treatment with multimodal drugs that interact with these mechanisms. In addition to revascularization, a successful future stroke therapy should include reduction of tPA-related side effects, prevention of cell death, stimulation of neuroregeneration, and plasticity (Rogalewski et al., 2006).

A number of neuroprotective combinations have been used with some success in animal models. These include the co-administration of an NMDA receptor antagonist with GABA receptor agonists, free radical scavengers, cytidine-5'-diphosphocholine, the protein synthesis inhibitor cyclohexamide, caspase inhibitors, or growth factors such as basic fibroblast growth factor (bFGF) (Singhal et al., 2006). Synergy is also observed with two different antioxidants, tirlazad mesylate and magnesium, (Schmid-Elsaesser et al., 1999), and cytidine-5'-diphosphocholine plus bFGF (Schäbitz et al., 1999). Caspase inhibitors given with bFGF or an NMDA receptor antagonist extend the therapeutic window and lower effective doses (Ma et al., 2001).

Synergistic or additive effects have also been reported when thrombolysis was used with neuroprotectants such as oxygen radical scavengers, AMPA and NMDA receptor antagonists, MMP inhibitors, cytidine-5'-diphosphocholine, topiramate, antileukocytic adhesion antibodies and antithrombotics (Rogalewski et al., 2006). Combination therapies may decrease dosages for each agent, thereby reducing the occurrence of adverse events. Two recent clinical trials have reported the feasibility and safety of treating with intravenous tPA followed by the neuroprotectants clomethiazole (Lyden et al., 2001) or lubeluzole (Grotta, 2001).

Another novel approach to consider for future treatment of ischemic stroke is to evaluate drugs with both neuroprotective and recovery-enhancing properties (Fisher, 2011). Such a drug combination would demonstrate robust effects on reducing infarct size and improving functional outcome when initiated shortly after stroke onset in animals. Such experimental data of tissue salvage when a drug is given early after experimental stroke onset and improved recovery without a reduction of infarct volume when the drug is initiated later are available. Both cytidine-5'-diphosphocholine and granulocyte colony stimulating factor (GCSF), two drugs currently in advanced clinical trials, have robust preclinical data that confirm early neuroprotection and late recovery enhancement effects (Clark, 2009; Minnerup et al., 2008). This research is ongoing.

In addition, human serum albumin therapy was introduced recently for the treatment of ischemic stroke (Ginsberg et al., 2011). Extensive animal studies have shown albumin in moderate to high doses to be a promising neuroprotectant in focal and global cerebral ischemia (Belayev et al., 2001; Belayev et al., 1999). In focal ischemia, albumin treatment diminished total infarct volume by two-thirds and reduced brain edema by three-fourths or more, with a therapeutic window of efficacy extending to 4 h; ameliorated brain swelling; improved blood flow to critically perfused brain regions; enhanced microvascular perfusion; reduced postischemic microvascular

THE STROKE PENUMBRA IS A TRANSLATIONAL TARGET

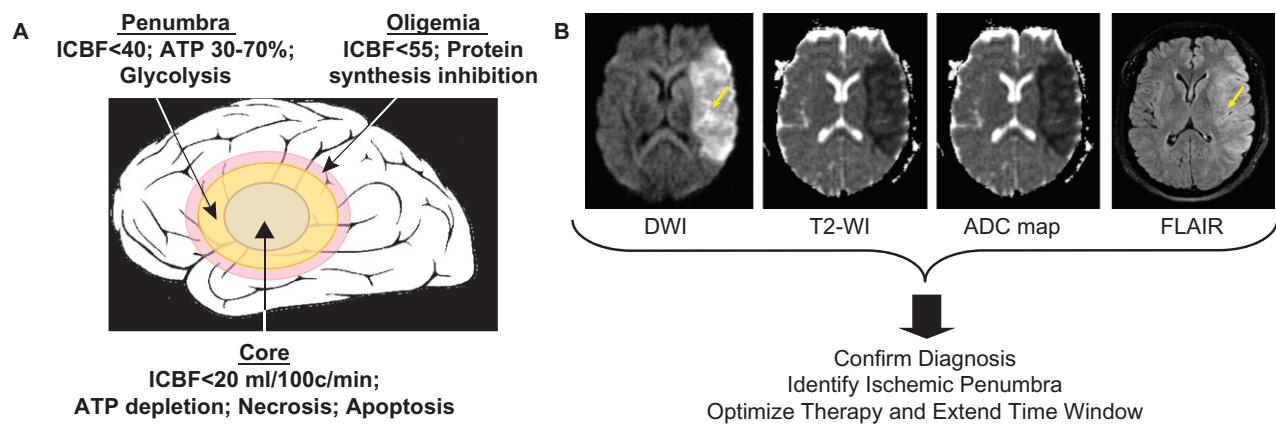
Tiffany N. Eady, Ludmila Belayev, Nicolas G. Bazan

Therapies for acute stroke have yielded very limited success in clinical trials. The current target of acute stroke therapy is to salvage the ischemic penumbra. The ischemic penumbra is a region surrounding the ischemic core that maintains some blood flow supplied by collateral circulation, and therefore survives the initial perfusion deficit. Regrettably, the penumbra often progresses to infarction over time with irreversible damage advancing from the region of the most severe blood flow reduction to the peripheral regions with less disturbed perfusion. This progression of damage is characterized by a complex cascade of electrophysiological, molecular, metabolic and perfusion disturbances. Although reduced local cerebral blood flow (LCBF) is a major factor responsible for necrotic injury, other events, including lipid peroxidation, inflammatory responses and brain edema, may contribute to either the severity or progression of penumbral injury. Thus, ischemia in the penumbra causes dysfunctions, but not severe enough ones to result immediately in irreversible damage. Prompt restoration of adequate perfusion in the penumbra by injection of thrombolytic agents may slow down the onset of irreversible damage in this area, thus limiting neurological deficit.

The window of opportunity for salvaging the penumbra is very short. Restoration of the blood supply can reduce more extensive brain tissue injury by salvaging reversibly damaged penumbral tissue. One therapeutic modality that has shown efficacy is thrombolysis using either intravenous or intra-arterial infusion of tissue plasminogen activator (r-tPA). This mechanism provides a rationale for clinical trials, which have demonstrated that reperfusion after thrombolysis improves clinical outcome in selected patients with acute stroke. Neuroprotection strategies are novel experimental therapies that aim to diminish blood-brain barrier (BBB) breakdown and polymorphonuclear (PMN) activation in the parenchyma, accelerate inflammatory resolution, and downregulate apoptotic cell death. In animal models, intravenously infused docosahexaenoic acid (DHA) provides

an expanded time window of penumbra protection with neurological recovery after middle cerebral artery occlusion (MCAO) (Belayev et al., 2011).

Imaging the penumbra is a new strategy for detecting tissue at risk. It has been reported that roughly half of all acute ischemic patients still have penumbral tissue 18 hours after stroke on magnetic resonance imaging (MRI), and as such these areas are potentially salvageable. Yet, due to comorbid conditions and contraindications, only 8% of all ischemic stroke patients are eligible for treatment with r-tPA. The remaining patients have potentially salvageable tissue with no medical means to increase the chance of survival. The penumbra has traditionally been studied by positron emission tomography (PET) and single photon emission computed tomography (SPECT), but recently magnetic resonance imaging (MRI) techniques have also allowed us to gain insight into the mechanisms underlying ischemia. Cerebral blood flow techniques such as PET and SPECT have been shown to correlate with clinical outcome and have been used with success for clinical trials. More recently, the hypoxic ligand ^{18}F -fluoromisonidazole (FMISO) and the neuronal receptor ligand ^{11}C -flumazenil (FMZ) have been used to refine the capability of PET to identify the penumbra. The use of ^{18}F -FMISO PET with quantitative three-dimensional mapping of the penumbra in acute ischemic stroke patients, grouped by time since stroke onset, demonstrates a central to peripheral evolution of infarction with eventual loss of the penumbra. CT perfusion has great promise as a diagnostic tool due to its ability to deliver reliable perfusion maps accurately. The passage of the contrast agent through the brain can be recorded, and parametric maps of cerebral blood volume and flow, as well as contrast mean transit time, can be generated. Using these imaging modalities to identify the ischemic penumbra is an important next step in extending the therapeutic time window beyond five hours and providing acute stroke therapy to those patients most likely to respond (see figure below).



Panel A: Drawing showing ischemic core and penumbra. Blood flow reduction causes metabolic disturbances at certain blood flow thresholds. The ischemic core has depleted ATP levels while the penumbra has a gradient reduction of ATP between normal/oligemic tissue and the ischemic core. **Panel B:** Diffusion-weighted image (DWI), T2-weighted image (T2-WI), apparent diffusion coefficient (ADC) and fluid-attenuated inversion recovery (FLAIR) showing a hyperintense lesion and edema (bright regions; yellow arrows) 18 hours after stroke (Courtesy of Karen A. Tong, M.D., Loma Linda University, CA).

blood element adhesion; and helped to transport important free fatty acids to the postischemic brain (Belayev et al., 2005; Belayev et al., 2001; Belayev et al., 2002). An 82-subject pilot-phase dose escalation trial has shown that albumin is safe, with strong preliminary suggestions of possible efficacy (Palesch et al., 2006). The albumin in acute stroke (ALIAS) multicenter phase III clinical trial is under way in 50 clinical sites in the United States and Canada (Ginsberg et al., 2011).

Acknowledgments

We thank Laura L. Dugan and Jeong Sook Kim-Han, the authors of this chapter in the seventh edition, which provided an exceptionally rich support for the current chapter.

References

- Aarts, M., Liu, Y., Liu, L., et al. (2002). Treatment of ischemic brain damage by perturbing NMDA receptor- PSD-95 protein interactions. *Science*, 298, 846–850.
- Agre, P., King, L. S., Yasui, M., et al. (2002). Aquaporin water channels—from atomic structure to clinical medicine. *Journal of Physiology*, 542, 3–16.
- Aktan, A. O., & Yalcin, A. S. (1998). Ischemia-reperfusion injury, reactive oxygen metabolites, and the surgeon. *Turkish Journal of Medical Sciences*, 28, 1–5.
- Allen, C. L., & Bayraktutan, U. (2009). Oxidative stress and its role in the pathogenesis of ischaemic stroke. *International Journal of Stroke*, 4, 461–470.
- Bano, D., Young, K. W., Guerin, C. J., et al. (2005). Cleavage of the plasma membrane Na⁺/Ca²⁺ exchanger in excitotoxicity. *Cell*, 120, 275–285.
- Bazan, N. G. (2005). Neuroprotectin D1 (NPD1): A DHA-derived mediator that protects brain and retina against cell injury induced oxidative stress. *Brain Pathology*, 15, 159–166.
- Bazan, N. G. (2009). Neuroprotectin D1-mediated anti-inflammatory and survival signaling in stroke, retinal degenerations, and Alzheimer's disease. *Journal of Lipid Research*, 50, S400–S405.
- Belayev, L., Bustó, R., Zhao, W., et al. (1996). Quantitative evaluation of blood-brain barrier permeability following middle cerebral artery occlusion in rats. *Brain Research*, 739, 88–96.
- Belayev, L., Khoutorova, L., Atkins, K. D., et al. (2009). Robust docosahexaenoic acid-mediated neuroprotection in a rat model of transient, focal cerebral ischemia. *Stroke*, 40, 3121–3126.
- Belayev, L., Khoutorova, L., Atkins, K. D., et al. (2011, March). Docosahexaenoic acid therapy of experimental ischemic stroke. *Translational Stroke Research* (In press).
- Belayev, L., Liu, Y., Zhao, W., et al. (2001). Human albumin therapy of acute ischemic stroke: Marked neuroprotective efficacy at moderate doses and with a broad therapeutic window. *Stroke*, 32, 553–560.
- Belayev, L., Marcheselli, V. L., Khoutorova, L., et al. (2005). Docosahexaenoic acid complexed to albumin elicits high-grade ischemic neuroprotection. *Stroke*, 36, 118–123.
- Belayev, L., Pinard, E., Nallet, H., et al. (2002). Albumin therapy of transient focal cerebral ischemia: *In vivo* analysis of dynamic microvascular responses. *Stroke*, 33, 1077–1084.
- Belayev, L., Saul, I., Huh, P. W., et al. (1999). Neuroprotective effect of high-dose albumin therapy against global ischemic brain injury in rats. *Brain Research*, 845, 107–111.
- Borsig, T., Clarke, P. G., Hirt, L., et al. (2003). A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia. *Nature Medicine*, 9, 1180–1186.
- Clark, W. M. (2009). Efficacy of citicoline as an acute stroke treatment. *Expert Opinion on Pharmacotherapy*, 10, 839–846.
- Connolly, E. S., Winfree, C. J., Springer, T. A., et al. (1996). Cerebral protection in homozygous null ICAM-1 mice after middle cerebral artery occlusion. Role of neutrophil adhesion in the pathogenesis of stroke. *Journal of Clinical Investigation*, 97, 209–216.
- Dankbaar, J. W., Hom, J., Schneider, T., et al. (2010). Dynamic perfusion-CT assessment of early changes in blood-brain barrier permeability of acute ischaemic stroke patients. *Journal of Neuroradiology*.
- de Groot, H., & Rauen, U. (2007). Ischemia-reperfusion injury: Processes in pathogenetic networks: A review. *Transplantation Proceedings*, 39, 481–484.
- del Zoppo, G. J. (2006). Stroke and neurovascular protection. *The New England Journal of Medicine*, 354, 553–555.
- del Zoppo, G. J., Schmid-Schönbein, G. W., Mori, E., et al. (1991). Polymorphonuclear leukocytes occlude capillaries following middle cerebral artery occlusion and reperfusion in baboons. *Stroke*, 22, 1276–1283.
- Dobbin, J., Crockard, H. A., & Ross-Russell, R. (1989). Transient blood-brain barrier permeability following profound temporary global ischaemia: An experimental study using ¹⁴C-AIB. *Journal of Cerebral Blood Flow and Metabolism*, 9, 71–78.
- Ebinger, M., De Silva, D. A., Christensen, S., et al. (2009). Imaging the penumbra—strategies to detect tissue at risk after ischemic stroke. *Journal of Clinical Neuroscience*, 16, 178–187.
- Faulkner, J. R., Herrmann, J. E., Woo, M. J., et al. (2004). Reactive astrocytes protect tissue and preserve function after spinal cord injury. *The Journal of Neuroscience*, 24, 2143–2155.
- Fisher, M. (2006). The ischemic penumbra: A new opportunity for neuroprotection. *Cerebrovascular Diseases*, 2, 64–70.
- Fisher, M. (2011). New approaches to neuroprotective drug development. *Stroke*, 42, S24–S27.
- Fiskum, G., Murphy, A. N., & Beal, M. F. (1999). Mitochondria in neurodegeneration: Acute ischemia and chronic neurodegenerative diseases. *Journal of Cerebral Blood Flow and Metabolism*, 19, 351–369.
- Ginsberg, M. D., Palesch, Y. Y., Martin, R. H., et al. (2011). The albumin in acute stroke (ALIAS) multicenter clinical trial: Safety analysis of part 1 and rationale and design of part 2. *Stroke*, 42, 119–127.
- Grotta, J. (2001). and combination therapy stroke trial investigators. combination therapy stroke trial: Recombinant tissue-type plasminogen activator with/without lubeluzole. *Cerebrovascular Diseases*, 12, 258–263.
- Haddad, G. G., & Jiang, C. (1997). O₂-sensing mechanisms in excitable cells: Role of plasma membrane K⁺ channels. *Annual Review of Physiology*, 59, 23–42.
- Hall, A. A., & Pennypacker, K. R. (2010). Implications of immune system in stroke for novel therapeutic approaches. *Translational Stroke Research*, 1, 85–95.
- Hall, E. D., Andrus, P. K., Smith, S. L., et al. (1996). Neuroprotective efficacy of microvascularly localized versus brain-penetrating antioxidants. *Acta Neurologica Supplementum*, 66, 107–113.
- Halliwell, B. (1992). Reactive oxygen species and the central nervous system. *Journal of Neurochemistry*, 59, 1609–1623.
- Hardingham, G. E., & Bading, H. (2010). Synaptic versus extra-synaptic NMDA receptor signaling: Implications for neurodegenerative disorders. *Nature Reviews Neuroscience*, 11, 682–696.
- Heiss, W. D. (2010). The concept of the penumbra: Can it be translated to stroke management?. *International Journal of Stroke*, 5, 290–295.
- Hossmann, K. M. A. (2009). Pathophysiological basis of translational stroke research. *Folia Neuropathologica*, 47, 213–227.
- Jin, R., Yang, G., & Li, G. (2010). Molecular insights and therapeutic targets for blood-brain barrier disruption in ischemic stroke: Critical role of matrix metalloproteinases and tissue-type plasminogen activator. *Neurobiology of Disease*, 38, 376–385.

- Kleindorfer, D., Kissela, B., Schneider, A., et al. (2004). Eligibility for recombinant tissue plasminogen activator in acute ischemic stroke: A population-based study. *Stroke*, 35, 27–29.
- Kloska, S. P., Wintermark, M., Engelhorn, T., et al. (2010). Acute stroke magnetic resonance imaging: Current status and future perspective. *Neuroradiology*, 52, 189–201.
- Kristian, T., & Siesjo, B. K. (1997). Changes in ionic fluxes during cerebral ischaemia. *International Review of Neurobiology*, 40, 27–45.
- Krupp, J. J., Vissel, B., Thomas, C. G., et al. (1999). Interactions of calmodulin and α -actinin with the NR1 subunit modulate Ca^{2+} -dependent inactivation of NMDA receptors. *Journal of Neuroscience*, 19, 1165–1178.
- Lee, J. K., Kwak, H. J., Piao, M. S., et al. (2010). Quercetin reduces the elevated matrix metalloproteinases-9 level and improves functional outcome after cerebral focal ischemia in rats. *Acta Neurologica*.
- Lipton, S. A. (2006). Paradigm shift in neuroprotection by NMDA receptor blockade: Memantine and beyond. *Nature Reviews Discovery*, 5, 160–170.
- Lloyd-Jones, D., Adams, R., Carnethon, M., et al. (2009). Heart disease and stroke statistics—2009 update: A report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation*, 119, e21–e181.
- Lo, E. H., Broderick, J. P., & Moskowitz, M. A. (2004). tPA and proteolysis in the neurovascular unit. *Stroke*, 35, 354–356.
- Lo, E. H., Dalkara, T., & Moskowitz, M. A. (2003). Mechanisms, challenges and opportunities in stroke. *Nature Reviews Neuroscience*, 4, 399–415.
- Lucas, S. M., Rothwell, N. J., & Gibson, R. M. (2006). The role of inflammation in CNS injury and disease. *British Journal of Pharmacology*, 147(Suppl. 1), s232–s240.
- Lukiw, W. J., Cui, J. G., Marcheselli, V. L., et al. (2005). A role for docosahexaenoic acid-derived neuroprotectin D1 in neural cell survival and Alzheimer disease. *Journal of Clinical Investigation*, 115, 2774–2783.
- Lyden, P., Jacoby, M., Schim, J., et al. (2001). The Clomethiazole Acute Stroke Study in tissue-type plasminogen activator-treated stroke (CLASS-T): Final results. *Neurology*, 57, 1199–1205.
- Ma, J., Qiu, J., & Hirt, L., et al. (2001). Synergistic protective effect of caspase inhibitors and bFGF against brain injury induced by transient focal ischaemia. *British Journal of Pharmacology*, 133, 345–350.
- Marcheselli, V. L., Hong, S., Lukiw, W. J., et al. (2003). Novel docosanoids inhibit brain ischaemia-reperfusion-mediated leukocyte infiltration and pro-inflammatory gene expression. *The Journal of Biological Chemistry*, 278, 43807–43817.
- Markus, R., Reutens, D. C., Kazui, S., et al. (2004). Hypoxic tissue in ischaemic stroke: Persistence and clinical consequences of spontaneous survival. *Brain*, 127, 1427–1436.
- Mehta, S. L., Manhas, N., & Raghuram, R. (2007). Molecular targets in cerebral ischemia for developing novel therapeutics. *Brain Research Reviews*, 54, 34–66.
- Minnerup, J., Heidrich, J., Wellmann, J., et al. (2008). Meta-analysis of the efficacy of granulocyte-colony stimulating factor in animal models of focal cerebral ischemia. *Stroke*, 39, 1855–1861.
- Molina, C. A. (2011). Reperfusion therapies for acute ischemic stroke: Current pharmacological and mechanical approaches. *Stroke*, 42, S16–S19.
- Moskowitz, M. A., Lo, E. H., & Iadecola, C. (2010). The science of stroke: Mechanisms in search of treatments. *Neuron*, 67, 181–198.
- Nakka, V. P., Gusain, A., Mehta, S. L., et al. (2008). Molecular mechanisms of apoptosis in cerebral ischemia: Multiple neuroprotective opportunities. *Molecular Neurobiology*, 37, 7–38.
- Nicholls, D., & Attwell, D. (1990). The release and uptake of excitatory amino acids. *Trends in Pharmacological Sciences*, 11, 462–468.
- Oku, N., Kashiwagi, T., Hatazawa, J., et al. (2010). Nuclear neuroimaging in acute and subacute ischemic stroke. *Annals of Nuclear Medicine*, 24, 629–638.
- Palesch, Y. Y., Hill, M. D., Ryckborst, K. J., et al. (2006). The ALIAS Pilot Trial: A dose-escalation and safety study of albumin therapy for acute ischemic stroke—II: Neurologic outcome and efficacy analysis *Stroke* (37).
- Pan, J., Konstas, A. A., Bateman, B., et al. (2007). Reperfusion injury following cerebral ischemia: Pathophysiology, MR imaging, and potential therapies. *Neuroradiology*, 49, 93–102.
- Pulsinelli, W. (1992). Pathophysiology of acute ischaemic stroke. *Lancet*, 339, 533–536.
- Ramos-Cabrer, P., Campos, F., Sobrino, T., et al. (2011). Targeting the Ischemic Penumbra. *Stroke*, 42, 7–11.
- Ramos-Fernandez, M., Bellolio, M. F., & Stead, L. G. (2011). Matrix metalloproteinase-9 as a marker for acute ischemic stroke: A systematic review. *Journal of Stroke and Cerebrovascular Diseases*, 20, 47–54.
- Ringelstein, E. B., & Nabavi, D. G. (2005). Cerebral small vessel diseases: Cerebral microangiopathies. *Current Opinion in Neurology*, 18, 179–188.
- Rivers, C. S., Wardlaw, J. M., Armitage, P. A., et al. (2006). Do acute diffusion- and perfusion-weighted MRI lesions identify final infarct volume in ischemic stroke? *Stroke*, 37, 98–104.
- Rodriguez de Turco, E. B., Belayev, L., Liu, Y., et al. (2002). Systemic fatty acid responses to transient focal cerebral ischemia: Influence of neuroprotectant therapy with human albumin. *Journal of Neurochemistry*, 83, 515–524.
- Rogalewski, A., Schneider, A., Ringelstein, E. B., et al. (2006). Toward a multimodal neuroprotective treatment of stroke. *Stroke*, 37, 1129–1136.
- Rothwell, N. J., & Luheshi, G. N. (2000). Interleukin 1 in the brain: Biology, pathology and therapeutic target. *Trends in Neurosciences*, 23, 618–625.
- Sage, J. I., Van Uitert, R. L., & Duffy, T. E. (1984). Early changes in blood brain barrier permeability to small molecules after transient cerebral ischemia. *Stroke*, 15, 46–50.
- Sandoval, K. E., & Witt, K. A. (2008). Blood-brain barrier tight junction permeability and ischemic stroke. *Neurobiology of Disease*, 32, 200–219.
- Schäbitz, W. R., Li, F., Irie, K., et al. (1999). Synergistic effects of a combination of low-dose basic fibroblast growth factor and citicoline after temporary experimental focal ischemia. *Stroke*, 30, 42731.
- Schmid-Elsaesser, R., Hungerhuber, E., Zausinger, S., et al. (1999). Neuroprotective efficacy of combination therapy with two different antioxidants in rats subjected to transient focal ischemia. *Brain Research*, 816, 471–479.
- Schreiber, J., Eling, T. E., & Mason, R. P. (1986). The oxidation of arachidonic acid by the cyclooxygenase activity of purified prostaglandin H synthase: Spin trapping of a carbon-centered free radical intermediate. *Archives of Biochemistry and Biophysics*, 249, 126–136.
- Serhan, C. N., Yacoubian, S., & Yang, R. (2008). Anti-inflammatory and proresolving lipid mediators. *Annual Review of Pathology*, 3, 279–312.
- Sheng, H., Bart, R. D., Oury, T. D., et al. (1999). Mice overexpressing extracellular superoxide dismutase have increased resistance to focal cerebral ischemia. *Neuroscience*, 88, 185–191.
- Simard, J. M., Sahuquillo, J., Sheth, K. N., et al. (2010). Managing malignant cerebral infarction. *Current Treatment Options in Neurology*.
- Simon, R. P., Swan, J. H., & Meldrum, B. S. (1984). Blockade of N-methyl-D-aspartate receptors may protect against ischemic damage in the brain. *Science*, 226, 850–852.
- Simopoulos, A. P. (2008). The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Experimental Biology and Medicine (Maywood)*, 233, 674–688.
- Singhal, A., Lo, E., Dalkara, T., et al. (2006). Ischemic Stroke: Basic Pathophysiology and Neuroprotective Strategies.: *Acute ischemic stroke. Part 1*. New York: Springer-Verlag Berlin Heidelberg.

- Smith, W. S. (2006). Safety of mechanical thrombectomy and intravenous tissue plasminogen activator in acute ischemic stroke. Results of the multi Mechanical Embolus Removal in Cerebral Ischemia (MERCI) trial, part I. *AJNR American Journal of Neuroradiology*, 27, 1177–1182.
- Sugawara, T., & Chan, P. H. (2003). Reactive oxygen radicals and pathogenesis of neuronal death after cerebral ischemia. *Antioxidants & Redox Signaling*, 5, 597–607.
- Thomas, C. G., Miller, A. J., & Westbrook, G. L. (2006). Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons. *Journal of Neurophysiology*, 95, 1727–1734.
- Trotti, D., Rizzini, B. L., Rossi, D., et al. (1997). Neuronal and glial glutamate transporters possess an SH-based redox regulatory mechanism. *The European Journal of Neuroscience*, 9, 1236–1243.
- Tu, W., Xu, X., Peng, L., et al. (2010). DAPK1 interaction with NMDA receptor NR2B subunits mediates brain damage in stroke. *Cell*, 140, 222–234.
- Van Eldik, L. J. (2002). Structure and enzymology of a death-associated protein kinase. *Trends in Pharmacological Sciences*, 23, 302–304.
- Wang, J., Liu, S. H., Fu, Y. P., et al. (2003). Cdk5 activation induces CA1 pyramidal cell death by direct phosphorylation of NMDA receptors. *Nature Neuroscience*, 6, 1039–1047.
- Ward, R. E., Huang, W., Curran, O. E., et al. (2010). Docosahexaenoic acid prevents white matter damage after spinal cord injury. *Journal of Neurotrauma*, 27, 1769–1780.
- Weiss, N., Miller, F., Cazaubon, S., et al. (2009). The blood–brain barrier in brain homeostasis and neurological diseases. *Biochimica et Biophysica Acta*, 1788, 842–857.
- Welch, K. M. A., Caplan, L. R., Reis, D. J., Siesjo, B. K., & Weir, B. (Eds.), (1997). *Primer on cerebrovascular diseases*. San Diego, CA: Academic Press.
- White, B. C., Sullivan, J. M., DeGracia, D. J., et al. (2000). Brain ischemia and reperfusion: Molecular mechanisms of neuronal injury. *Journal of Neurological Sciences*, 179, 1–33.
- Xiong, Z. G., Zhu, X. M., Chu, X. P., Minami, M., Hey, J., Wei, W. L., et al. (2004). Neuroprotection in ischemia: Blocking calcium-permeable acid-sensing ion channels. *Cell*, 118, 687–698.
- Yamashita, T., Deguchi, K., Nagotani, S., et al. (2010). Vascular protection and restorative therapy in ischemic stroke. *Cell Transplant*
- Zador, Z., Stiver, S., Wang, V., et al. (2009). Role of aquaporin-4 in cerebral edema and stroke. *Handbook of Experimental Pharmacology*, 190, 159–170.
- Zhang, L., Zhang, Z. G., Liu, X., et al. (2006). Treatment of embolic stroke in rats with bortezomib and recombinant human tissue plasminogen activator. *Thrombosis and Haemostasis*, 95, 166–173.
- Zhang, R. L., Chopp, M., Chen, H., et al. (1994). Temporal profile of ischemic tissue damage, neutrophil response, and vascular plugging following permanent and transient (2H) middle cerebral artery occlusion in the rat. *Journal of Neurological Sciences*, 125, 3–10.
- Zou, Y., Chen, C. H., Fike, J. R., et al. (2009). A new mouse model for temporal- and tissue-specific control of extracellular superoxide dismutase. *Genesis*, 47, 142–154.