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Handbook of Neurochemistry and Molecular Neurobiology

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Acute Ischemic Injury and Repair in the Nervous System

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Abel Lajtha (Ed.)

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Volume Editor: Pak H. Chan

With 55 Figures and 16 Tables

Springer

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Preface

Stroke is a global health problem affecting approximately 15 million people annually in the world and about 700,000 people in the USA. It is the third leading cause of death and the most common cause of disability in most developed countries. The majority of strokes are ischemic (the blood flow to the brain is blocked), whereas a small percentage of strokes are hemorrhagic (the blood vessels burst). The molecular and cellular mechanisms of neuronal death that lead to brain injury and infarction after ischemic stroke have been intensively investigated over the past several decades. These investigations have led to our current understanding of the neurobiology of the disease at the molecular and cellular levels and have provided an impetus for the development of therapeutic strategies to treat brain injury in patients after an ischemic stroke.

This volume is intended to provide state-of-the-art and the most up-to-date knowledge on the mechanisms of neuronal death and repair after stroke. It begins with an overview of gene expression profiling in ischemic brain injury and ischemic tolerance for identification of the genes/proteins that are involved in neuronal death and neuroprotection. This chapter is followed by major discussions by leading experts in the field on the chosen gene/protein candidates that may affect neuronal death and survival. These candidate proteins include apoptotic-inducing factor (AIF) and poly(ADP-ribose) polymerase-1 (PARP-1), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), endothelial nitric oxide synthase (eNOS), and neuronal nitric oxide synthase (nNOS). Activation or expression of these proteins is known to induce DNA damage that leads to neuronal apoptosis. A succinct overview of the mechanisms of DNA damage and repair in ischemic neurons follows.

Protein synthesis, folding, aggregation, and degradation are known to play major roles in mediating the fate of ischemic neurons and this aspect is discussed, followed by a chapter that describes the neuroprotective or apoptotic role of heat shock protein. The neurobiology of superoxide dismutase, the key endogenous antioxidant enzyme in free radical detoxification and signaling in neuronal death or survival after cerebral ischemia is reviewed, and this basic research provided mechanistic support to the recent success of spin trap NXY-059 in a clinical stroke trial.

The neurovascular unit, which consists of many cell types, and its interaction and integrity have recently evolved as prime targets for therapeutic intervention in stroke. Thus, the two chapters that describe the biology of glial and cerebral endothelial cells and their role in the integrity and repair of the neurovascular unit are of major importance. This is followed by reviews on sex steroids and gender in ischemic pathobiology and the mechanisms of ischemic cell death in the developing brain. A review of the molecular mechanisms of cell death following subarachnoid hemorrhage is presented. The basic molecular and cellular mechanisms of neuroprotection of the antibiotic minocycline, a potential therapeutic agent in clinical stroke, are clearly reviewed by experts in the field. This chapter is followed by the concluding chapter, an outstanding review of the roles of matrix metalloproteinases and tissue plasminogen activator in reperfusion therapy that target the neurovascular unit in stroke.

It is our belief that this volume provides an excellent review of the tremendous advances of the past decades in neurochemical and molecular biological aspects of cerebral ischemia. We hope that these advances, as communicated through this volume, will provide an impetus for basic scientists and clinicians to further their translational research and to promote the insights for development of therapeutic interventions for stroke.

Pak H. Chan, Ph.D.

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1 Gene Expression Profiling in Ischemic Brain Injury and Ischemic Tolerance

C. A. Harrington · S. Stevens · M. Stenzel-Poore · R. P. Simon

Abstract: Potent endogenous mechanisms of neuroprotection are encoded in the genome, and the expression of a subset of these genes helps to determine whether cells survive ischemia. A genomic approach may identify and characterize these genes and the neuroprotective pathways through which their protein products operate. Identification of gene products that are endogenous neuroprotectants contributes significantly to our understanding of the pathophysiology of ischemic neuronal injury and would point the way toward new therapeutic approaches to stroke and related disorders such as traumatic brain injury. Here we review a strategy for discovering neuroprotective genes in ischemia by the use of mouse models of ischemic tolerance and microarray analysis to identify genes that are transcriptionally regulated in tolerance. We provide Affymetrix microarray analysis of an ischemic tolerance data set and review in detail the approach to genomic screening.

1 Introduction

Modulation of gene expression affects stroke outcome. The upregulation of apoptosis effector genes is induced in vulnerable neurons following ischemic injury (Chen et al., 1996b), but cells surviving ischemia express neuroprotective genes (Chen et al., 1995, 1996a). Thus the process of ischemia and particularly its severity affects brain survival and does so in substantial measure by way of transcription and translational regulation. This is most clearly demonstrated by brief periods of ischemia producing the phenomenon of "ischemic tolerance." This endogenous program of neuroprotection requires protein synthesis as protein synthesis inhibitors block tolerance induction (Barone et al., 1998). Gene products produced during the development of tolerance have been identified. Upregulation of the immediate early genes (IEG) (c-fos, c-jun, junB, and junD) occurs after preconditioning ischemia, however, the genes targeted by these transcription factors in the setting of tolerance are unknown (An et al., 1993; Abe and Nowak, 1996). The transcription factor hypoxia inducible factor-1 (HIF-1) is involved in preconditioning in the neonatal rat brain (Bergeron et al., 2000; Bernaudin et al., 2002); Akt has been implicated in this model as well (Bergeron et al., 2000; Bernaudin et al., 2002). Antioxidant enzymes, such as MnSOD, are overexpressed in tolerant brain and may play a role in protection (Ohtsuki et al., 1992; Kato et al., 1995). The induction of heat-shock proteins (HSPs) follows a time course compatible with tolerance (Chen et al., 1996a). Transgenic mice overexpressing HSP70 have attenuated ischemic injury (Rajdev et al., 2000). However, tolerance of cortical neurons to subsequent global ischemia occurs in the absence of HSP mRNA expression when cortical spreading depression is used as a preconditioning stimulus. While preconditioning in the gerbil induces IEG expression, tolerance develops in the absence of an increase in HSP72 mRNA levels (Kobayashi et al., 1995; Abe et al., 1996).

The antiapoptotic Bcl-2 family proteins are candidate mediators of tolerance. We have used antisense techniques to inhibit Bcl-2 induction in ischemia (Chen et al., 2000). Rats treated with Bcl-2 antisense oligodeoxynucleotides (ODN) prior to ischemia develop larger cerebral infarcts than untreated rats. Rats treated prior to preconditioning ischemia do not upregulate Bcl-2 protein and do not become tolerant to subsequent ischemic challenge (Shimizu et al., 2001). Bcl-2 is also increased in CA 1 sector neurons in global ischemic tolerance (Shimazaki et al., 1994). Thus, expanding the picture of gene regulation in ischemic brain from predictable effectors to the genomic response broadly is likely to be revealing.

2 Gene Expression Profiling

The introduction of DNA microarrays in the mid-1990s provided a tool for measuring transcription levels from thousands of genes in a single assay, a feat not previously achievable. Use of microarrays (also called chips) for gene expression profiling has led to an explosion in data on the transcriptome of a wide variety of cells and tissues. Global expression studies on the brain and other nervous system tissues are allowing neurological systems and perturbations of normal function due to injury or disease to be better understood.

Microarray expression-profiling technology relies on high-density arraying of defined DNA sequences on solid surfaces to which labeled cDNA or cRNA is hybridized. Gene transcript levels are monitored by

measurement of the fluorescent signals emitted by hybridized nucleic acids. DNA microarrays thus expand the bounds of expression analysis; with a typical microarray one can screen 10,000 or more different gene transcripts at one time (e.g., the Affymetrix GeneChip Murine Genome U74 Set, composed of three high-density oligonucleotide arrays representing over 36,000 full-length mouse genes and expressed sequence tag (EST) clusters). Recent progress in microarray fabrication technology has resulted in arrays in which an entire eukaryotic genome can be represented on a single chip. There are two basic types of microarray technology in wide use today: the Affymetrix GeneChip oligonucleotide array (Lipshutz et al., 1999) and the spotted DNA microarray (Ferea and Brown, 1999); the former has been employed in the work presented here and is described in more detail below.

2.1 GeneChip Technology

Affymetrix has adapted technology from the electronics industry to develop a photolithographic protocol for synthesis of oligonucleotides anchored to glass. Synthetic linkers carrying photolabile blocking groups are attached to the glass surface. A photolithographic mask directs light to those areas of the glass slide where the first nucleotide is to be attached. This light removes the blocking groups in these areas and nucleotides are added to the substrate where the blocking group is missing. Each oligonucleotide carries the same blocking group, so building a collection of chains requires only four masks at each base-addition step along the oligonucleotide. This union of photolithography and combinatorial chemistry allows the fabrication of tens of thousands of different oligonucleotides in relatively few synthesis steps in a highly ordered array on the chip surface. As per the nomenclature used for Northern analysis, these oligonucleotides are called probes. On GeneChip expression arrays, each gene is represented by 11–20 probes, each 25 nucleotides long. Each probe occupies a tile on the GeneChip array, with 10^6 and 10^7 probe copies per tile. For each gene-specific probe tiled on the array, there is a corresponding probe with a single, central base mismatch. This mismatch probe, built on an adjacent tile, operates as a control for nonspecific hybridization to the cognate sequence. Therefore, (11–20) $\times$ 2 probes, the "probe set," interrogate each transcript. Hybridization targets for the chip assay are generated by reverse transcription and amplification of isolated messenger RNA (mRNA) or total cellular RNA (cRNA) to produce biotinylated antisense RNA. Following array hybridization, the labeled cRNA bound to probes on the array is stained with fluorescent dye and detected using a laser scanner. A weighted average hybridization intensity value for each transcript is determined utilizing the individual probe pairs for each probe set, where PM_i is the intensity of a tile with a perfectly matching oligonucleotide, MM_i is the intensity of the adjacent tile with a mismatched oligonucleotide. Probe intensity values are used to determine relative levels of gene expression within individual samples and difference in expression levels of the same gene between samples. Production of GeneChip arrays is a proprietary process; only premade chips are available, and these can be obtained only from Affymetrix.

2.2 Microarray Assay

Expression profiling is performed on either total or poly A+ RNA extracted from the samples of interest. Sample preparation and hybridization for the GeneChip array assay is performed using standardized protocols provided by the manufacturer. mRNA is converted to double-strand cDNA; the cDNA is then used as template in an in vitro transcription reaction to generate multiple copies of antisense RNA labeled with biotin. The labeled RNA (target) is added to hybridization solution and injected into a cartridge containing a fluid chamber in contact with the probe surface of the selected GeneChip expression array. After hybridization, biotinylated targets annealed to probes on the array are stained with streptavidin-phycoerythrin. Array hybridization and processing are performed on equipment available from Affymetrix. The equipment comprises a hybridization oven, fluidics station, confocal laser scanner, and Windows-based computer. The fluidics station manages the washing and staining of each array following overnight hybridization with target; the distribution of hybridized fluorescent targets on the array is imaged using

the scanner. The resultant image is examined with the Affymetrix Microarray Suite (MAS) software. Expression profiles are determined using expression algorithms available in MAS. These analyses produce information about which gene transcripts are present in a given sample and which genes show differences in expression levels between samples. Further analyses may be performed by examining the data with other statistical and visualization strategies as described in *Sect. 5.1*.

3 Microarray Studies in Ischemia

We hypothesized that application of high-density microarrays to gene expression profiling of tolerant and ischemic brain would provide revealing information about how tolerance is established and maintained and how it modulates the brain's response to ischemia. Focal ischemia induces gene changes in ipsilateral cortex and preconditioning focal ischemia reduces ischemic infarction in the ipsilateral cortex. This injury and protection can be modeled in rodents and studied at the genomic level, and the differential expression of genes induced by the preconditioning versus ischemic stimulus elucidated. Differences between these expression profiles should identify novel protective mediators and define pathways for therapeutic interventions.

Brains of mice 24 or 72 h following middle cerebral artery occlusion (MCAO) were obtained in three different conditions: (1) preconditioning alone (15-min MCAO), (2) injurious ischemia alone (60-min MCAO), and (3) preconditioning followed 72 h later by injurious ischemia. Mice were perfused with heparinized saline to remove cells in the microcirculation, and the ischemic and nonischemic hemispheres were isolated separately. Tissue RNA was purified from the region of the cortex that, in the absence of preconditioning, is damaged by injurious ischemia while in the presence of preconditioning is protected from injury. Three pools of RNA were generated within each group by combining cortices from two to three mice.

High-density DNA microarray analyses (Affymetrix, Inc. Santa Clara, California) were performed using the murine genome U74A (MG-U74Av1) chip. Expression data were analyzed using MAS version 4.0 expression analysis algorithm to generate an average intensity value for each probe set (gene) and an "expression call" of either Absent (not detectable) or Present. Regulated genes were identified based on the following two criteria: (1) a >2.2-fold difference of expression in the ipsilateral hemisphere as compared to the contralateral hemisphere in at least two of the three pools of RNA; (2) a p value of less than 0.05 using a t-test, comparing differences between hemispheres. A gene was considered differentially regulated if both conditions were met. The stringent criteria allowed for a higher confidence in the genes identified as regulated. Genes given a call of Absent on all chips were excluded from the analysis.

Our studies revealed that of the ~7,500 genes interrogated, 267 genes showed differential regulation either 24 or 72 h after preconditioning alone, injurious ischemia, or preconditioning followed by injurious ischemia compared to the nonischemic control hemisphere. A similar number of genes were modulated significantly in each of the three ischemic conditions 24 h after MCAO (*Figure 1-1*) as well as 72 h after MCAO (data not shown). Surprisingly, very few of the regulated genes were common to more than one of the three experimental conditions at 24 h (*Figure 1-2*); no genes were common to all conditions, whereas two genes were modulated in both preconditioning and injurious ischemia alone, and five genes were common to injurious ischemia and preconditioning plus injurious ischemia (*Figure 1-2*).

In addition to the minimal sharing of differentially expressed genes among the three conditions, we also found that the pattern of gene expression (upregulation versus down regulation) was different in the various settings. Specifically, ischemic preconditioning alone and injurious ischemia cause a pronounced *upregulation* of gene expression while preconditioning preceding injurious ischemia results in marked *downregulation* of gene expression. Modulation of gene expression following injurious ischemia revealed that of the genes regulated, 49 (86%) were *increased* in expression compared to the nonischemic hemisphere (*Figure 1-3*). In contrast, preconditioning preceding injurious ischemia resulted in decreased expression in 47 (77%) of the regulated genes (*Figure 1-3*). This decrease was not a generalized phenomenon as housekeeping genes, such as GAPDH and actin, are not differentially regulated and 23% of the genes regulated in this condition are increased. This suggests that a prior stimulus of preconditioning alters the transcriptional response to injurious ischemia via a specific pattern of gene suppression.

Figure 1-1

Number of genes regulated 24 h following preconditioning alone, injurious ischemia alone, or preconditioning followed by injurious ischemia. Inclusion of a gene as regulated was based on expression criteria described in text

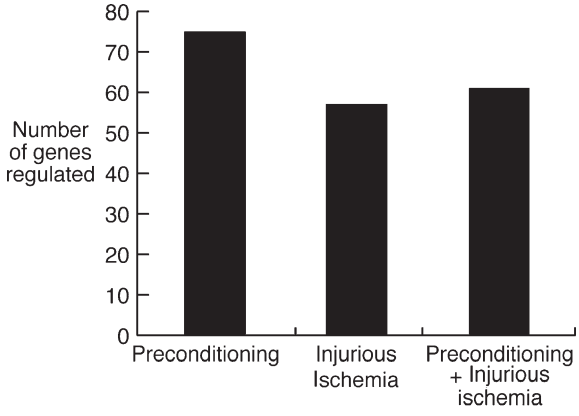
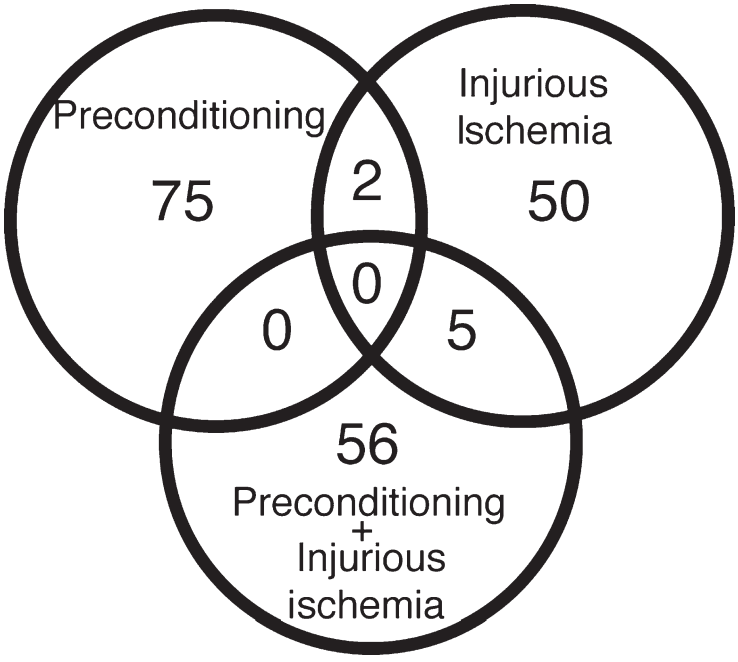


Figure 1-2

Venn diagram showing the number of genes regulated in the three conditions at 24 h



Analysis of expression patterns according to gene function using the Mouse Genome Database (2002a), the Stanford-Source Web site (2002b), and literature review shows genes of unknown function represent ~30% of the response in each experimental condition. Further the response to injury by damaging ischemia is dominated by upregulation of genes that coordinate immune responses and host defense (36% of genes with identified function; indicated in the category of defense) (*Figure 1-4*). This contrasts with the

Figure 1-3

The number of genes increased or decreased in the ischemic hemisphere as compared to the nonischemic hemisphere

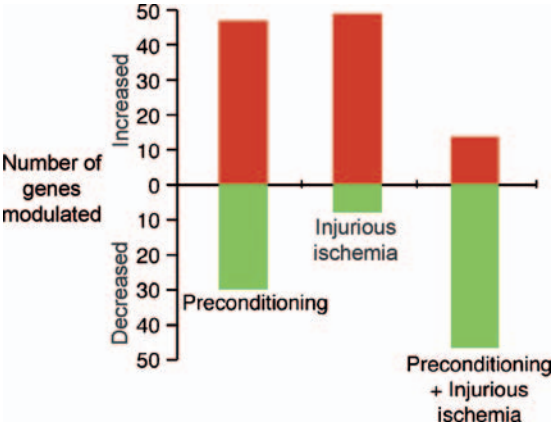
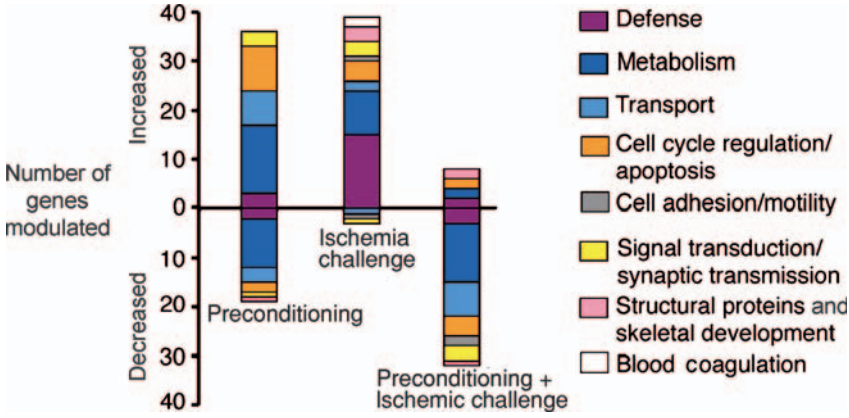


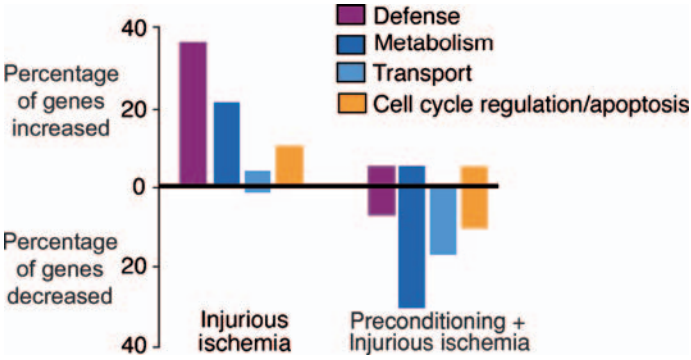
Figure 1-4

A putative assignment of gene function was determined based on information obtained from the Mouse Genome Database (2002a), the SOURCE Web site (2002b), and published literature. Data shown does not include genes in which no functional information was available



response to preconditioning alone, wherein the global gene expression pattern is predominated by increased expression of genes involved in metabolic functions that affect cellular homeostasis (25%; indicated in the category of metabolism) (*Figure 1-4*) and regulation of the cell cycle (16%). The most striking of the gene profiles is the response to ischemic injury following preconditioning. In this setting, the overall transcriptional response to injury is downregulated and the majority of the genes suppressed involve pathways that regulate metabolism, molecular transport, or cell cycle control (30%, 17%, and 10% of genes with identified function, respectively) (*Figure 1-4*). Thus, the response to injury in the context of preconditioning is biased toward dampened metabolism, suppressed molecular transport, and restricted cell cycling. Moreover, preconditioning stimulus *reprograms* the subsequent response to ischemic injury. A comparison of a subset of genes regulated in injurious ischemia shows that the nature of the response is altered dramatically by prior preconditioning (*Figure 1-5*). The response to ischemic injury in the absence of preconditioning exhibits heightened expression of genes required for defense and repair [e.g.,

Figure 1-5
A comparison of the gene regulation in the four most common functional categories



cyclooxygenase-2 (Cox-2); complement components; HSPs 10, 70, and 105], whereas preconditioning reprograms the response to ischemic injury such that only a minor component involves defense and repair, while the net effect is reduced metabolic activity and transport (e.g., *N*-acetyl galactosaminidase, carboxyl ester lipase, alcohol dehydrogenase, potassium voltage-gated channel Kv1.5, kainate receptor, Doc2beta). The preponderance of gene suppression in protected brain holds promise for translational strategies. The therapeutic correlate suggested would be pharmacologic blockade of effector molecules to recapitulate neuroprotection.

In tolerant brain, changes are seen in genes that would be predicted to induce metabolic suppression, which is consistent with the fact that oxygen limitation leads to decreased energy utilization due, in part, to decreased demand (Hochachka et al., 1996). Several genes are suppressed that would be expected to influence cellular energetics via alterations in protein utilization, ion permeability, and energy metabolism. Suppression of these genes may decrease energy demands and conserve energy. Whether preconditioning alters the metabolic state of the brain challenged with injurious ischemia as suggested by these gene profiles requires further study. However, our microarray analyses revealed that several genes involved in ion transport, including the voltage-gated potassium channel subunit Kv1.5, showed decreased expression in the brains given preconditioning followed by injurious ischemia consistent with our in vitro functional studies; in collaboration with Zhigang Xiong at the Dow Neurobiology Labs, we have shown that potassium ion channel function and whole cell conductance are reduced by preconditioning (Stenzel-Poore et al., 2003). In summary, the transcriptional response to preconditioning alone or preconditioning followed by injurious ischemia reveals a profile that suggests suppression of cellular energy use and attenuation of ion channel activity. Such features may contribute to neuroprotection during limited oxygen availability. The genomic response to injurious ischemia is that of upregulation of transcription, particularly, the one associated with defense/immunity and metabolism. Of interest is the modest upregulation of genes regulating apoptosis and the cell cycle (*Figure 1-4*), areas which have been the focus of major research efforts regarding the cell biology of ischemic injury.

3.1 Post Hoc Analyses of Genomic Expression Patterns

Verification of our microarray results was performed using real-time polymerase chain reaction (PCR) (Taqman ABI 7700) shown in *Table 1-1*. By this post hoc analysis, approximately 80% of the genes identified as regulated (2.2-fold change, $p < 0.05$) by microarray analysis were indeed regulated. On the basis of these results, the false positive rate of detection is quite low (8%), which fits with the relatively stringent regulation criteria. At the protein level using Western blot or immunohistochemistry, levels for osteopontin (OPN), TNFRp55, HSP70, Cox-2, Cox-1 and GFAP were increased consistent with the gene expression measurements.

Table 1-1
Post hoc analysis of microarray results using real-time PCR

	Precond 24 h		Precond 72 h		Challenge 24 h		Challenge 72 h		Precond + Challenge 24 h	
	Array	RTPCR	Array	RTPCR	Array	RT PCR	Array	RT PCR	Array	RT PCR
Osteopontin	NC	I	I*	I	I	I	I	I	NC	I
GFAP	NC	NC	I	I	I	I	I	I	I	I
Calpactin	NC	I	I	I	I	NC	I	I	NC	NC
FXYD7	NC	NC	NC	NC	NC	NC	NC	D	D	D
AlcDhy	NC	D	NC	I	NC	I	NC	D	D	NC
NAGA	NC	NC	NC	I	NC	I	NC	D	D	NC
TNFRp55	NC	NC	NC	I	NC	I	I	I	NC	NC

Primers and probes (integrated DNA technologies) were designed based on the target sequences supplied by Affymetrix. β-Actin was used as the housekeeping gene and genes were quantitated in regards to a relative standard curve. A 1.7-fold change by real-time PCR was accepted as regulated. Yellow denotes false negative and blue denotes false positive
*Increased in the pilot experiment
NC no change, *I* increased, *D* decreased

4 Characterizing the Products of Regulated Genes

To test genes identified through microarray analysis regarding roles in the pathophysiology of cerebral ischemia and/or neuroprotection, we examined two potential candidate neuroprotectants—OPN and Cox-1.

4.1 Osteopontin

We discovered that the secreted glycoprotein OPN is regulated in ischemic tolerance (Meller et al., 2005). As it is a secreted protein that functions extracellularly, there is substantial therapeutic potential through available peptide analogs. We have performed basic characterization of this gene product that justifies advancing this molecule as a candidate mediator of neuroprotection. Specifically, we have performed post hoc analysis using real-time PCR to determine whether OPN is indeed upregulated in the ipsilateral cortex in preconditioning and ischemic challenge (*Table 1-1*). We have also found by Western blot (*Figure 1-6*) and immunohistochemistry (not shown) that OPN is upregulated in the ipsilateral hemisphere 72 h s/p 15-min (preconditioning) MCAO. These findings support OPN as a molecule whose regulation may be important in preconditioning. Importantly, we have found a pronounced neuroprotective effect of OPN in oxygen-glucose deprivation (OGD) (in vitro) and when administered to the brain (i.c.v) prior to MCAO in mice. These findings are very promising and bode well for our proposed strategy of gene selection and characterization of potential neuroprotective candidates.

4.2 Cox-1

We selected for further study, Cox-1, which is upregulated with preconditioning based on our microarray analysis. Cox-1 is a mediator known to be involved in ischemic protection, although not yet shown to be important in tolerance. We have performed basic characterization of Cox-1, as we did with OPN, and found by real-time PCR that mRNA expression of Cox-1 is upregulated following preconditioning (twofold). Protein expression levels are also increased in the cortex (ipsilateral) 72 h following ischemic preconditioning (15-min MCAO) (*Figure 1-7*). Immunohistochemical analyses to determine the identity and location in the CNS of Cox-1 positive cells (*Figure 1-8*) shows a nonneuronal localization.

Figure 1-6
OPN protein levels increased in the ipsilateral hemisphere 72 h following 15-min MCAO. α-Tubulin included as loading control

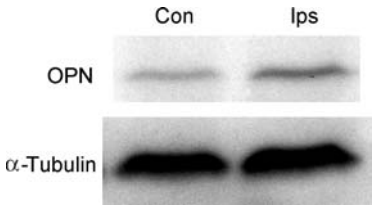


Figure 1-7
Cox-1 protein levels increased in the ipsilateral hemisphere 72 h following 15-min MCAO. α-Tubulin included as loading control

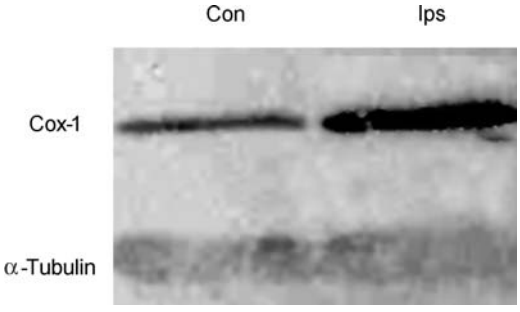
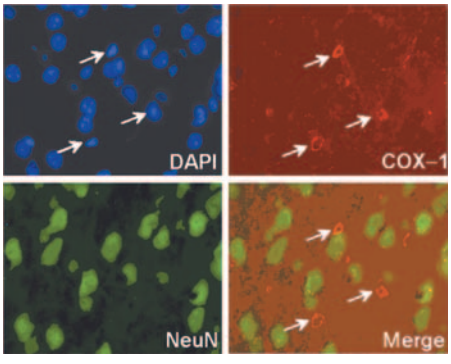


Figure 1-8
Cox-1 expression in nonneuronal cells in the mouse brain 72 h following 15-min MCAO. Brain sections from mice preconditioned with 15-min MCAO, 72 h prior to sacrifice were immunostained for Cox-1 and NeuN (a neuronal marker). No colocalization was detected. DAPI was used as a nuclear stain



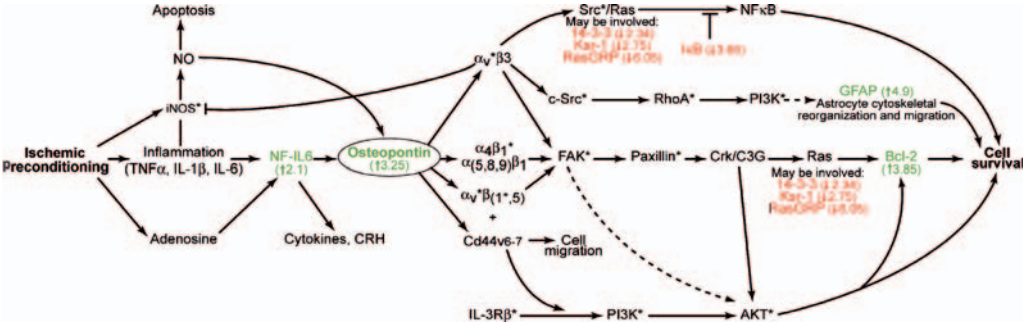
5 Elucidating Neuroprotective Pathways

The long-range goal of a genomic analysis is to identify pathways (in the case of ischemic tolerance, those leading to neuroprotection). To decipher potential pathways informatic approaches may be utilized (see data interpretation given later), but often pathways require the standard approach of using literature-based

information such as known actions, receptors, and cellular targets. Such information is derived from published studies and pathways in a variety of systems. We have created this type of theoretical pathway for OPN as a means of testing potential mechanisms of OPN's neuroprotective actions in the CNS (*Figure 1-9*). This pathway is based on the known receptors and second messengers that mediate OPN signaling in different biological settings.

Figure 1-9

OPN pathways involved in cell survival and cell migration. Ischemic preconditioning upregulates inflammatory mediators, such as TNFα, IL-1b, IL-6, which in turn regulate the transcription factor NF-IL6 leading to increased OPN expression. OPN may reside at a focal point in a network of several pathways operating through integrins that are known to transmit OPN signals and thereby influence cell survival. Examples of some of the known pathways involving OPN and cell survival are shown: (1) An early protective role of OPN may be mediated through its ability to downregulate iNOS (Guo et al., 2001) and reduce injury due to nitric oxide, (2) OPN is involved in endothelial cell survival through integrin-mediated NF-κB activation (Scatena et al., 1998), (3) Bcl-2, a known antiapoptotic gene is upregulated by OPN in kidney epithelial cells (Denhardt et al., 1995), (4) OPN induces cell migration in target cells through integrins or CD44 and has been shown specifically to induce migration of astrocytes and microglia to the site of glial scar formation (Ellison et al., 1998; Wang et al., 1998). Gray shows gene upregulation following 15 min preconditioning MCAO and 72-h recovery. Italics shows gene downregulation following 15 min preconditioning MCAO and 72-h recovery. ( ) Fold regulation over contralateral hemisphere. *Gene is represented on the microarray chip, however, not regulated within our experimental model



The use of genome-wide DNA microarrays provides us with a powerful tool that should allow us to define broad correlations of gene expression and create a portrait of transcriptional responses over time. Some of the genes induced are likely to be irrelevant to tolerance; others will countermand tolerance. However, an important subset should exist that forms a pathway leading to neuroprotection. This subset may comprise a consensus pathway—one that is the signature of an endogenous pathway of neuroprotection. Thus, pathway analysis could identify a conserved gene profile from tolerance and reveal genomic effectors of neuroprotection with significant potential for new therapeutic strategies in stroke and other forms of brain injury.

5.1 Technical Considerations

The ultimate measure of success in a study utilizing microarrays for expression profiling is the ability to use the information that is generated for identifying genes and pathways that impact the disease or biological system under study. In order to achieve this goal, the microarray experiment must measure transcripts at both high- and low-abundance levels and result in the detection of true differences in gene expression levels

among the samples tested. Accurate measurement requires that the expression profiles generated from each sample are reproducible and that results among replicate samples are consistent. The success of the overall study also depends on the selection of appropriate tools and strategies for the analysis of the array data once it is produced. In the following section, we describe our methods and strategies for successful microarray analysis and provide perspective on selecting effective approaches for data analysis and mining.

5.2 Overview

Each expression-profiling microarray assay requires the following steps: array selection or fabrication, RNA isolation, mRNA labeling, array hybridization and processing, image processing, and low-level data analysis. In subsequent steps, data from the multiple samples of an experimental group are normalized and further analyzed to determine patterns of gene expression and to identify differentially regulated genes.

The importance of bioinformatics and biostatistics in microarray analysis is the focus of several studies (Ermolaeva et al., 1998; Bassett et al., 1999; Zhang, 1999), which discuss the enormous quantity of data that need to be managed and analyzed, the complexity of data management and analysis, and the need to access existing biological knowledge of gene function. As illustrated by Ermolaeva et al. (1998), bioinformatics and biostatistics can make valuable contributions to each stage of a microarray experiment. At the preexperimental stage, information may be needed to identify genes and EST clones to be included in the array experiment, to select mRNA sources to form appropriate comparison groups, and to decide on the number of samples or experiments to be performed. At the experimental stage, image analysis requires the specification of methods for background and signal quantification, as well as background adjustment. At the postexperimental stage, data analysis is performed to identify genes that are differentially expressed between comparison groups, to classify genes with similar expression profiles, and finally, to interpret the resulting classification in terms of known gene functions.

Gene annotations and access to existing public databases are keys to translating statistical results into biologically meaningful hypotheses. The existing biological information can be used not only for the interpretation of the analysis results but can also drive the direction of the analysis and can be incorporated into the analysis itself. Thus, data integration is essential to both successful data analysis and interpretation. In general, data integration for the expression profiling study will include the following: gene expression data, statistical results, clinical and laboratory data, and public information on genes such as sequences, annotations, biological pathways, and functions.

5.3 Microarray Assay

Microarray assays with GeneChip arrays are performed using procedures adapted from the Affymetrix GeneChip Expression Analysis Technical Manual (http://www.affymetrix.com/support/technical/manual/expression_manual.affx).

5.3.1 Sample Preparation

The first step in the microarray assay is designed to produce sufficient levels of labeled mRNAs for hybridization and detection on the microarray. In the standard profiling assay, 3–4 μg of purified total RNA are amplified and labeled in a two-step procedure. In the first step, mRNA is converted to double-stranded cDNA using Superscript Reverse Transcriptase (Invitrogen, Carlsbad, CA) and an oligo-dT primer linked to a T7 RNA polymerase-binding site sequence (Integrated DNA Technologies, Coralville, IA). In the second step, amplified and biotin-labeled cRNA (the target) is produced in an in vitro transcription reaction using T7 RNA polymerase and biotinylated UTP and CTP (Enzo Diagnostics, Inc., Farmingdale, NY). Following removal of free nucleotides, target yield is measured by UV_{260} absorbance.

5.3.2 Array Hybridization

Labeled target is fragmented at 95°C in the presence of high magnesium concentration. The fragmented material is combined with control oligomer (used for grid alignment during image processing) and hybridization controls (Affymetrix, Inc., Santa Clara, CA) in hybridization buffer. About 10 µg of quality-tested target is then hybridized to the selected GeneChip microarray (Affymetrix, Inc.). Arrays are hybridized overnight, followed by washing, staining with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR), signal amplification with biotinylated anti-streptavidin antibody (Vector Labs, Burlingame, CA), and a final staining step. The distribution of fluorescent material on the processed array is determined using the GeneArray laser scanner (Affymetrix, Inc.).

5.3.3 Array Assay Quality Assessment

An important, although often overlooked, component of microarray profiling is quality control both before and after array hybridization. In our laboratory all samples are tested for assay performance quality prior to hybridization to the genome array of choice. In the study presented previously, 4 µg of target was hybridized overnight with a Test Array (Affymetrix, Inc.) and then washed and stained with streptavidin-phycoreythrin on the fluidics station. All GeneChip expression arrays, including the Test Array, contain control probe sets for both spiked and endogenous RNA transcripts (e.g., bacterial genes BioB, BioC, BioD, CreX, and species-specific "housekeeping" genes such as actin and GAPDH). Following image processing and absolute analysis of the array pattern with MAS (see *Sect. 5.4*), four values are examined: background, noise, average signal (or average difference, MAS 4.0), and ratio of signal values for probe sets representing the 5′ and 3′ ends of actin and GAPDH transcripts. In general, 3′/5′ ratios greater than 3 indicate that the target is overly biased to the 3′ end of the sample mRNAs, likely due to degraded input RNA. All targets that do not meet assay performance thresholds are remade whenever possible or discarded from further analysis.

We have recently updated our standard quality control procedures to incorporate a measurement of cRNA size distribution prior to array analysis. Using microfluidics separation technology, 200 ng of each labeled target is analyzed on an RNA NanoChip processed on the 2100 Bioanalyzer (Agilent, Inc., Palo Alto, CA). Samples that fail cRNA yield and size distribution thresholds are discarded prior to any array hybridization. Only borderline or atypical targets are applied to test arrays for quality assessment prior to hybridization to a GeneChip genome array.

The results of hybridization to each genome array are assessed in a manner similar to that used for the test array assay. A set of performance metrics are extracted from the MAS absolute analysis of each hybridization; these include background, noise, average signal, percent present, and ratio of signal values for probe sets representing the 5′ and 3′ ends of actin and GAPDH transcripts. Empirically defined thresholds for acceptable performance are used to exclude degraded samples or poorly performing assays from further analysis. These thresholds are usually specific to the array type used, with the exception of the 3′/5′ ratios for actin and GAPDH; 3 is the typical cutoff for all samples labeled with the one-cycle cDNA synthesis, in vitro transcription protocol.

5.4 Data Analysis

The analysis of data generated by the microarray expression-profiling experiment is a multistep process. The process can be broken down into three stages: preparation of raw data from individual arrays and normalization of data from experimentally grouped arrays; identification of significant gene changes or data patterns among samples; and interpretation of the results in light of known gene functions and physiological pathways. Underlying these steps in the analysis is a requirement for effective management and integration of large amounts of array data and associated gene and sample information.

5.4.1 Data Preparation

Data preparation involves image processing, normalization, transformation, and filtering. MAS is an Affymetrix proprietary software for image processing, signal quantification, background adjustment, preprocessing, scaling, and normalization. Further, MAS performs the analysis of a single GeneChip array (absolute analysis) as well as comparative analysis between two GeneChip arrays (comparative analysis). In the study described in this review, array hybridization patterns were processed using MAS version 4.0 software with the appropriate masking of the inaccurate probe sets for the version 1 MG-U74A chip. After image processing, array data were globally scaled to a uniform average intensity value prior to further analysis. The "empirical" expression analysis algorithm in MAS 4.0 (Lockhart and Barlow, 2001) utilizes a strategy in which hybridization intensity for the MM probe of each probe pair is subtracted from the PM probe in order to minimize the impact of background and cross-hybridization signals. The relative expression level of RNA transcripts for each gene represented on the array is calculated as the average difference intensity ($PM_i - MM_i$) for each probe set. The average difference intensity for each probe set is then compared to an empirically determined threshold value to assign a Call of Present or Absent (undetectable) for the respective gene transcript. Recently, a new expression algorithm has been introduced in MAS version 5.0 (MAS 5.0), which uses statistical tests to assign Present and Absent calls based on *p* values (Liu et al., 2002), and a one-step Tukey's biweight estimate with adjusted PM intensities to calculate the relative expression level (Signal) for each gene (Hubbell et al., 2002). Pairwise comparison analyses can also be performed with MAS software. In a comparison analysis, two samples hybridized to two GeneChip arrays of the same type are compared against each other to detect and quantify changes in gene expression. The comparison algorithm generates a change *p* value and an associated change call (increased, decreased, no change). The quantitative estimate of the gene expression change between samples is reported as a signal log ratio. MAS 5.0 algorithms have been verified empirically using a spiked "Latin Square" microarray data set.

MAS provides a scaling and normalization option for the single chip analysis and two-chip comparative analysis. A multiplicative factor is applied so that the overall mean intensity becomes equal to either the referent level (normalization) or arbitrary target intensity (scaling).

Alternative algorithms for preprocessing and normalizing array data have recently become available. These include several model based algorithms for probe-level data: the multiplicative model-based expression index (MBEI) (Li and Wong, 2001); see also DNA-Chip analyzer (dChip) (Schadt et al., 2001), the robust multi-array average (RMA) (Irizarry et al., 2003), and the position-dependent nearest neighbor (PDNN) analysis (Zhang et al., 2002).

5.4.2 Statistical Analysis and Data Exploration

In our initial strategy for the identification of differentially expressed genes, we use the calls and $\log_2$ ratio values produced by MAS analysis to select regulated genes. An alternative approach is to use graphical visualization to identify differentially expressed genes. These include two- and three-dimensional scatter plots of the signal intensity of two or three experimental conditions or ratios of two intensities among the treatment groups. Often, graphical analyses allow one to spot genes that appear as outliers, those that are differentially expressed. A formal statistical comparison is often challenging due to the small number of replications and a multiple comparison problem. However, there are several approaches that can be considered:

1. Analysis of variance (ANOVA) approach combined with a bootstrap analysis of the residuals (Kerr et al., 2000)
2. Empirical Bayesian analysis based on hierarchical modeling (Newton et al., 2000)
3. t-Statistics with adjusted permutation *p* values (Dudoit et al., 2000)
4. Significance analysis of microarrays (SAM) (Tusher et al., 2001)
5. Various nonparametric methods (Manduchi et al., 2000; Tsodikov et al., 2000)

Software programs employing these methods are in the public domain.

Many current data analysis techniques and software focus on graphical analyses (e.g., scatter plots and histograms) to identify genes that are differentially expressed between two comparison groups, cluster analysis (using algorithms such as hierarchical clustering and self-organizing maps) to classify genes with similar expression profiles, and graphical display of resulting clusters as a dendrogram (Eisen et al., 1998). Other statistical techniques successfully applied to microarray gene expression studies include principal component (Hilsenbeck et al., 1999), classification and regression tree (CART) (Dudoit et al., 2000), multidimensional scaling (Khan et al., 1998), singular value decomposition (Holter et al., 2000), and neural network (Brown et al., 2000) analyses. Michaels et al. (1998) discussed the application of cluster analysis and graphical visualization methods to time-series data. Recently, there were attempts to include prior biological knowledge of genes when clustering gene expression data (Loftus et al., 1999; Brown et al., 2000; Furey et al., 2000). In particular, Brown et al. (2000) proposed the use of support vector machines (SVMs), which contain functional classes of the genes as supplementary information to gene expression data, and developed a neural network algorithm that performed better than algorithms that did not use supplementary information. There are several new approaches to clustering: coupled two-way clustering analysis (Getz et al., 2000), plaid models for a two-way clustering (Lazzeroni and Owen, 2000), and gene shaving (Hastie et al., 2000), which combines principal component analysis with cluster analysis. There are several computer packages specifically designed for the statistical analysis of microarray gene expression studies, including Cluster and Tree View (Eisen et al., 1998), ArrayDB (NHGRI), GeneSight (BioDiscovery), GeneSpring (Silicon Genetics), MAExplorer (Lemkin et al., 2000), Rosetta Resolver (Rosetta BioSoftware), Interaction Explorer/ArrayAssist (Stratagene), DecisionSite for Functional Genomics (Spotfire). These programs provide various methods for graphical analysis, cluster analysis, principal component analysis, and unsupervised neural network clustering, while some also provide links to external data sources such as GenBank, Entrez, UniGene, and KEGG pathway views. Many standard statistical software packages, such as SAS, S-PLUS, SPSS, STATISTICA, and CART, can perform all or some of the statistical methods mentioned earlier.

5.5 Data Interpretation

One of the greatest challenges in bioinformatics and biostatistics posed by gene expression analysis is to identify methods both for the interpretation of the statistical results and for the use of these results to delineate possible biological or physiological pathways. Such tasks require integration of existing biological knowledge from public data sources such as GenBank, NCBI's Entrez system, PubMed, and MEDLINE. UniGene and KEGG pathway views both at the analysis stage and at the interpretation stage. Some efforts have been already made in this area. For example, GeneCards (http://nciarray.nci.nih.gov/cards/) and MedMiner (Tanabe et al., 2000) were developed by the NCI's bioinformatics group to assist further data mining and interpretation. Many software packages, such as GeneSpring and Rosetta Resolver, also provide built-in links to GenBank and BLAST search. Affymetrix maintains a web-accessible resource (NetAffx Analysis Center) that allows researchers to link their GeneChip array results with probe array design information and gene annotation information (Liu et al., 2003; Cheng et al., 2004). Such existing biological information can be used subsequently to define class classification of unknown ESTs that are clustered together with known genes using SVMs.

To facilitate the linkage of gene function to sequence information, the Gene Ontology (GO) Consortium has been developing a highly structured, controlled vocabulary of terms describing gene roles and gene products. This ontology is not organism specific and is adaptive to the rapidly changing field of functional genomic research. The GO is composed of three categories: (1) biological components to which the gene contributes, (2) molecular function of a gene, and (3) cellular location where a gene product is active. Genes and gene products are made up of these three attributes. Recognizing each of these as independent attributes, and using this information to annotate gene expression data, a contextual meaning can be placed on expression experiment results.

The terms defined by GO make up nodes of a network. The nodes make up parent–child relationships, which are relationships that are well defined within the network. The entire network makes up a directed

acyclic graph. It is adaptive to new terms being added and can be used to automatically update databases based on the GO ontology (The Gene Ontology Consortium, 2000). The GO terms, definitions, and gene associations are available for download in flat file format from the GO Web site (http://www.geneontology.org).

The UniGene collection is a database composed mostly of EST sequences. These sequences and genes are grouped in clusters to create a mapping used to remove redundancy and identify all the coding sequences within a genome (Schuler, 1997). EST sequences and genes may be retrieved from UniGene using queries composed of text, keyword, and accession number. Complete builds of the UniGene collection are available via file transfer protocol (FTP) from the UniGene Web site (http://www.ncbi.nlm.nih.gov/UniGene/) and are updated on a regular basis.

The LocusLink database organizes information around genes and creates a centralized repository for researchers to retrieve gene-specific information (Pruitt and Maglott, 2001). Information contained in LocusLink is composed of descriptive information about loci such as nomenclature, map positions, associations with disease, and accession numbers. Information can be retrieved using text-based queries for keywords, gene symbols, and accession numbers. LocusLink data is available via FTP from the LocusLink Web site (http://www.ncbi.nlm.nih.gov/LocusLink/) and partial updates are available on a daily basis.

5.6 Data Integration

Efficient use of gene expression data requires it to be stored in a relational format with relevant clinical, experimental, and biological data. The relational format allows investigators to search or cluster gene information based on both expression values and other biological information. This supplemental information, obtained from the relational database, helps support the statistical analyses we have described. Once relationships and patterns are identified through statistical analysis and contextually placed with supplemental data from the relational databases, links to data from public genomic databases can be generated to further aid in developing biological interpretations of the results.

5.6.1 Integration of Gene Function Classification with Microarray Data

Genes and ESTs used as targets on microarrays are identified using gene accession numbers. These accession numbers can be linked to UniGene cluster identifiers. The UniGene cluster identifiers provide a link to corresponding LocusLink records. On the basis of the GO accession number found in LocusLink, the GO functional classifications (biological, molecular, cellular) can be extracted and used to annotate the genes of interest identified during the analysis of expression experiment data.

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2 Neurobiology of AIF and PARP in Cerebral Ischemia

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Abstract: Polyadenosine diphosphoribose polymerase-1 (PARP-1) is an important mediator of neuronal cell death following cerebral ischemia. Normally a guardian of the genome required for the proper cellular response to DNA damage following mild to moderate stress, this nuclear enzyme is overactivated following acute neuronal injury. The result is unregulated synthesis of polyadenosine diphosphoribose (PAR) followed by widespread neuronal cell death. Once thought to be a purely necrotic type of cell death, recent findings suggest that one underlying mechanism of this PARP-1-dependent neuronal cell death appears to involve the translocation of the mitochondrial flavoprotein apoptosis-inducing factor (AIF) to the nucleus, which triggers a caspase-independent form of cell death. Inhibition of PARP-1 activity results in decreased PAR synthesis, inhibition of AIF translocation, and extensive neuronal cell survival. Therefore, PARP-1 and AIF, two principal mediators of neuronal cell death following cerebral ischemia, are emerging as therapeutic targets in the treatment of stroke.

List of Abbreviations: AIF, apoptosis-inducing factor; NMDA, *N*-methyl-D-aspartate; PARP-1, poly(ADP-ribose) polymerase-1; OGD, oxygen-glucose deprivation; H-I, hypoxia-ischemia; MCAO, middle cerebral artery occlusion; cyt *c*, cytochrome *c*; PAR, polyadenosine diphosphoribose; PARG, poly(ADP-ribose) glycohydrolase

1 Introduction

Stroke, or focal ischemia, is a leading source of morbidity and mortality in the USA. It is the third leading cause of death and disability in the USA, and to date no therapeutic regimens are available to completely deter the harm caused by this acute condition, which involves the cessation of normal blood flow through neuronal tissue. In actuality, much of the current treatment protocols are supportive due to our limited understanding of the events following stroke. Currently, we know that the cellular signaling events following focal ischemia are complex with several identified protein targets and pathways contributing to neuronal injury secondary to the primary infarct. However, one pathway has been identified that serves as a primary mediator of excitotoxic neuronal cell death following cerebral ischemia: poly(ADP-ribosyl)ation. Activation of *N*-methyl-D-aspartate (NMDA) receptors by glutamate and the subsequent production of nitric oxide (NO) lead to the activation of polyadenosine diphosphoribose polymerase-1 (PARP-1) through DNA damage. Disruption of poly(ADP-ribosyl)ation through the specific inhibition of PARP-1 provides the greatest amount of neuroprotection following cerebral ischemia in experimental animal models shown to date. Further, central to this mechanism is the translocation of apoptosis-inducing factor (AIF), a conserved mitochondrial flavoprotein, to the nucleus. The ability of AIF to serve as the death effector in this pathway is dependent on PARP-1 activation. Thus, AIF and PARP-1 are recently identified mediators of neuronal injury in stroke and other neurodegenerative conditions. AIF and PARP-1 are emerging as novel therapeutic targets for the treatment of stroke. This article reviews the experimental findings regarding excitotoxicity, poly(ADP-ribosyl)ation, and AIF translocation in neuronal cell death following cerebral ischemia.

2 Excitotoxicity, Nitric Oxide, and PARP-1

2.1 Excitotoxic Neuronal Cell Death

The damage following stroke is manifest through several neurotoxic events. Primary neuronal injury occurs via oxygen and nutrient deprivation. Further, secondary injury occurs due to metabolic dysfunction and oxidative stress, which may result in severe neuronal damage (Dawson and Dawson, 1997). Excitotoxicity is thought to be a major trigger for neuronal injury following stroke. One pathway that is involved in excitotoxic cell death is DNA damage secondary to peroxynitrite formation. Glutamate is a major mediator of this secondary neuronal damage, in large part, through activation of NMDA receptors (Choi, 1994). The result is increases in intracellular calcium and activation of calcium-dependent enzymes, including NO synthase (NOS), the formation NO, and elevated levels of superoxide anion. This generation of NO and free radicals plays a key role in the excitotoxic damage induced by focal ischemia (Samdani et al., 1997). In addition, superoxide and NO react to form the highly reactive peroxynitrite, which induces DNA damage, thereby causing even more devastation (Szabo and Dawson, 1998).

Other pathways also contribute to neuronal injury following insult. Postsynaptically, glutamate interacts with several different receptors (Dani and Mayer, 1995). Stimulation of these receptors may also contribute to stroke-related injury. However, since much of the damage following cerebral ischemia is due to excitotoxic neuronal injury, this review will focus on the NMDA receptor, the generation of NO, and activation of poly(ADP-ribosyl)ation.

2.2 Poly(ADP-ribosyl)ation

Multicellular organisms possess a unique and ubiquitous biochemical pathway: the metabolism of adenosine diphosphoribose (ADP-ribose) polymers. These polymers are synthesized by the PARPs using nicotinamide adenine dinucleotide (NAD^+) as substrate with the subsequent release of nicotinamide (Amé et al., 2000) (*Figure 2-1*). Nuclear proteins are acceptors of these polymers, which results in their covalent modification (Amé et al., 2000). Polyadenosine diphosphoribose (PAR) varies in size, with the largest composed of up to 200 ADP-ribose units with multiple branch points (Alvarez-Gonzalez and Jacobson, 1987). They are highly negatively charged and provide higher affinity binding sites for DNA-binding proteins (Althaus et al., 1995). This covalent modification is only transient due to the rapid action of PAR glycohydrolase (PARG), which catalyzes the hydrolysis of PAR into free ADP-ribose (Amé et al., 2000) (*Figure 2-1*). The short duration of PAR polymer half-life (Juarez-Salinas et al., 1979; Amé et al., 2000) suggests a closely coordinated modus operandi of PARP-1 and PARG, which also underscores the importance of both enzymes in this cyclic pathway of protein modification by poly(ADP-ribosyl)ation.

PAR synthesis and degradation is present in all mitotic and postmitotic cells with few exceptions (de Murcia and Shall, 2000). In the central nervous system (CNS), PARP and PARG are present throughout the brain and spinal cord. However, different cell types contain different levels of PAR metabolism as quantified by immunoblotting or immunocytochemistry. High levels of PARP-1 activation are seen in phagocytosing microglial cells but not in resting microglia (Ullrich et al., 2001). PARP activation is present in macroglial cells, most notably in astrocytes. Neurons have high levels of PARP-1 activity following ischemic injury, which explains the extreme sensitivity of certain neurons to PARP-1-dependent excitotoxic cell death mediated by the neurotransmitter glutamate (Yu et al., 2003). This observation provides the foundation for the ability of poly(ADP-ribosyl)ation to mediate cell death in the nervous system.

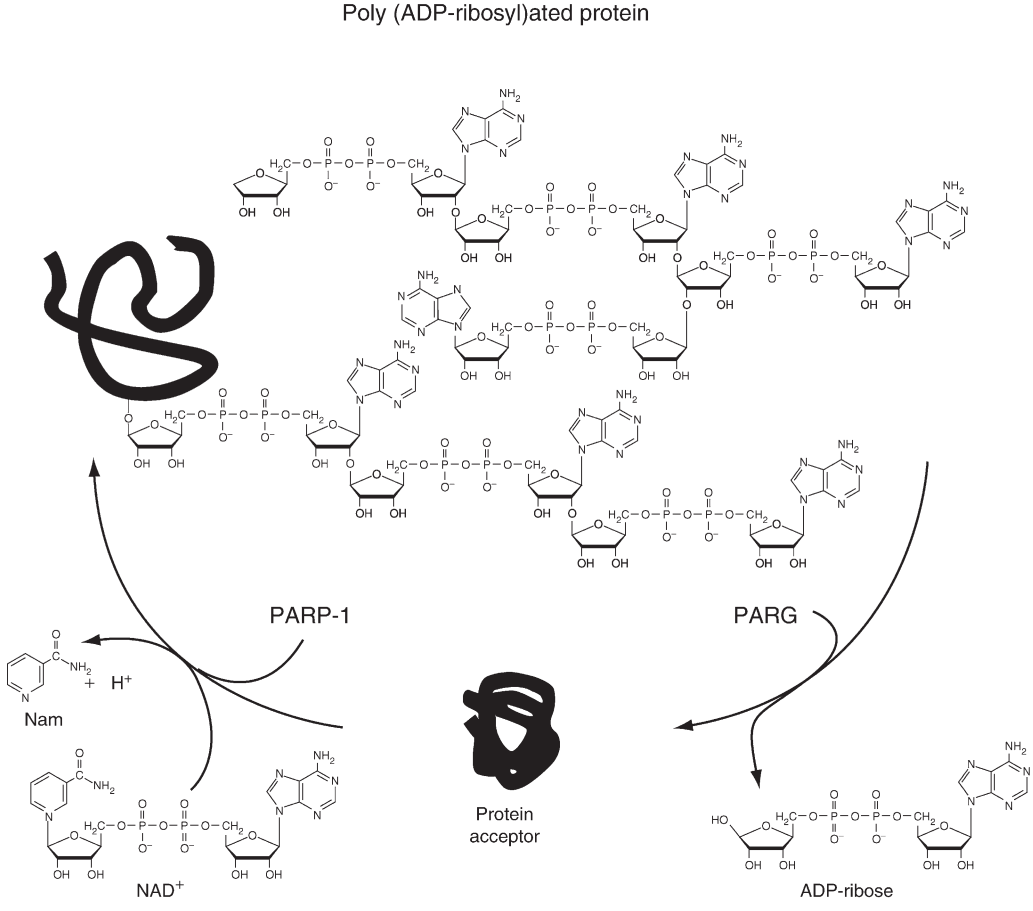
2.2.1 PARP-1

Features Poly(ADP-ribosyl)ation is an immediate cellular response to genomic damage (Amé et al., 2000). Most studies regarding this metabolism have focused on PARP-1 (EC2.4.2.30), the original and best understood PARP. It is specifically activated by DNA strand breaks to synthesize PAR (Amé et al., 2000). Basal levels of PAR are very low but in response to DNA-damaging agents 20- to 1000- fold increases in polymer levels are stimulated (Juarez-Salinas et al., 1979; Jacobson et al., 1983). Protein acceptors of this covalent modification include PARP-1 itself (Adamietz, 1987), DNA-binding proteins (Adamietz and Rudolph, 1984; Ruscetti et al., 1998; Simbulan-Rosenthal et al., 1999), and DNA repair proteins (Simbulan-Rosenthal et al., 1998). Consequently, poly(ADP-ribosyl)ation is critical for efficient DNA repair, since mice displaying the PARP-1-null genotype have been shown to be defective in DNA base-excision repair (Trucco et al., 1998).

Structural Biology The domain structure of this 113-kDa protein consists of a 42-kDa N-terminal DNA-binding domain containing a nuclear localization signal (NLS), a 16-kDa central automodification/BRCA1 C-terminal (BRCT) domain, and a 55-kDa C-terminal catalytic domain (Amé et al., 2000). To date, there are 18 other proteins that share homology to the PARP-1 catalytic domain (Menissier de Murcia et al., 2003). Most of the fully characterized members of this PARP family are similar in that they are specifically activated by DNA strand breaks, although several members do not require DNA for activity (Smith et al., 1998; Kickhoefer et al., 1999). They differ in their subcellular localizations and different capacities for generating PAR. PARP-1, a nuclear enzyme selectively activated by DNA damage and the most abundant and best understood PARP, is responsible for generating long-chained, branched PAR polymer

Figure 2-1

Poly(ADP-ribosyl)ation. Poly(ADP-ribose) (PAR) polymers are synthesized by the poly(ADP-ribosyl) transferase enzymes known as PAR polymerases (PARPs) using NAD^+ as substrate with the subsequent release of nicotinamide (Nam). Nuclear proteins are acceptors of the PAR, which results in their covalent modification. They can vary in size, with the largest containing up to 200 ADP-ribose residues with multiple branch points. However, the covalent modification of proteins by the transfer of ADP-ribose residues is only transient due to the rapid action of poly(ADP-ribose) glycohydrolase (PARG), which catalyzes the hydrolysis of these polymers into free ADP-ribose. An ADP-ribosyl protein lyase has been proposed to remove the protein-proximal ADP-ribose residue from the acceptor protein (not shown)



(Juarez-Salinas et al., 1979). PARP-2, PARP-3, and tankyrase, all thought to be DNA damage sentinels that facilitate DNA repair or maintain telomere length, generate short-chained PAR polymers (Smith et al., 1998; Amé et al., 1999; Augustin et al., 2003). It is not yet known if other PARPs contribute to neurotoxicity. Also, the functions of the remaining putative PARP family members are not yet known.

2.2.2 PARG

Features and Structural Biology The rapid synthesis and rapid degradation of PAR is an immediate response to DNA damage. This transient nature of polymers, which may attain a length of up to 200

residues, reflects the importance of PAR polymer catabolism as the complementary action to PARP. PARG (EC 3.2.1.143), the enzyme responsible for this action, catalyzes the hydrolysis of the O-linked $\alpha(1''\rightarrow2')$ or $\alpha(1'''\rightarrow2'')$ ribosyl-ribose linkages (Amé et al., 2000). As a result, the covalent modification of proteins by PAR is only transient due to the rapid action of PARG, which suggests a closely coordinated modus operandi for the PARPs and PARG. The bovine PARG cDNA encodes a 111-kDa protein that consists of a putative N-terminal regulatory domain containing an NLS (Meyer-Ficca et al., 2004), a central putative bipartite NLS, and a C-terminal catalytic domain (Lin et al., 1997). PARG is encoded by a single-copy gene (Lin et al., 1997), it shares no homology with any other known protein, and to date, no other PARG homologs have been discovered within the mammalian genome. Because PARG is unique, its cellular importance is paramount in restoring native protein structure and function and completing the PAR polymer cycles initiated by the entire PARP family. The hydrolysis of these polymers in all cases, however, appears to be similar since PAR is rapidly hydrolyzed by PARG once synthesized. An ADP-ribosyl protein lyase has been proposed to remove the final, protein-proximal ADP-ribose residue from the acceptor protein (Oka et al., 1984), although this protein has yet to be identified.

2.3 PARP-1 and Ischemic Neuronal Injury

2.3.1 PARP-1 and Cell Death

Poly(ADP-ribosyl)ation has long been associated with cell death due to the observation that PARP-1 is a target of caspases activated during apoptosis (Kaufmann et al., 1993). It is cleaved into a 24-kDa fragment containing the DNA-binding domain and an 89-kDa fragment containing the catalytic and automodification domains during apoptosis (de Murcia and Shall, 2000). The 24-kDa fragment may contribute to the irreversibility of apoptosis by blocking access of DNA repair enzymes to strand breaks (Amours et al., 2001) and thereby facilitate cellular disassembly. The cleavage of PARP-1 may also be critical in producing an 89-kDa fragment incapable of activation by DNA nicks, which suggests a possible mechanism to prevent energy depletion.

Because PARP-1 responds to DNA damage in a dose-dependent fashion, poly(ADP-ribosyl)ation can become very energetically expensive process due to the consumption of NAD^+, the substrate of PARP, during PAR synthesis. NAD^+ is required in the electron transport chain and is therefore essential for the synthesis of ATP in mitochondria. Accordingly, overactivation of PARP-1 is proposed to cause NAD^+ depletion. The cessation of energy metabolism ensues, which leads to the loss of all energy dependent cellular functions, resulting in cell death by necrosis (Berger, 1985).

2.3.2 Nitric Oxide, PARP-1, and Stroke

Neuronal damage following cerebral ischemia is primarily caused by the activation of the NMDA receptors and the formation of NO, superoxide anion, and ultimately peroxynitrite. The ability of glutamate to mediate this excitotoxicity via the formation of NO and the production of free radicals led to the discovery of PARP-1 activation secondary to this cascade. Increases in intracellular calcium via NMDA stimulation can activate a variety of enzymes, such as proteases, kinases, phospholipases, and NOS. Formation of NO via NOS ultimately results in the formation of peroxynitrite, which damages DNA and activates PARP-1, thereby initiating poly(ADP-ribosyl)ation (Zhang et al., 1994). The neurotoxicity elicited from ischemia is mediated by NO, which ultimately activates PARP-1 through DNA damage caused by the highly reactive peroxynitrite. PARP-1 inhibitors are neuroprotective against excitotoxicity and the magnitude of this neuroprotection is directly proportional to the potency of the inhibitor (Zhang et al., 1994). The most compelling evidence of this role of PARP-1 is seen in mice displaying the PARP-1-null genotype. Primary neuronal cultures from these mice are resistant to the toxicity elicited by combined oxygen-glucose deprivation (OGD) or by neurotoxic levels of NMDA or NO generators (Eliasson et al., 1997). Further, reduced infarct volume is observed following middle cerebral artery occlusion (MCAO) in the whole animal (Eliasson et al., 1997; Endres et al., 1997). Although significant neuroprotection is provided through the

overexpression of the superoxide dismutase or disruption of the neuronal NOS (nNOS) gene in mice following MCAO (Sampei et al., 2000), knockout of the PARP-1 gene provides the greatest magnitude of neuroprotection in vitro and in vivo. Thus, poly(ADP-ribosyl)ation through PARP-1 activation may be a "choke-point" for neuronal cell death programs.

2.3.3 Therapeutic Potential of PARP-1 Inhibition in the Treatment of Stroke

Cell death secondary to stroke and other ischemic conditions comprises the majority of neuronal injury, and therefore is the primary cause of the morbidity associated with these conditions. This neuronal damage is attributed, in part, to excitotoxicity due to the massive release of the excitatory neurotransmitter glutamate which acts on NMDA receptors and ultimately activates poly(ADP-ribosyl)ation. The generation of free radicals at the site of injury is also detrimental to tissue distal to the focal point due to reperfusion. In each condition, inhibitors of glutamate receptors, NOS inhibitors, and free-radical scavengers are shown to be neuroprotective, but the greatest neuroprotection observed to date in this type of neuronal injury model is the inhibition of PARP-1. Disruption of the functional PARP-1 gene confers resistance to the neuronal cell death elicited by in vitro models of ischemia, while PARP-1 null mice are protected against in vivo neuronal injury following MCAO, an in vivo model of stroke and reperfusion injury in the whole animal (Eliasson et al., 1997; Endres et al., 1997). Administration of different PARP-1 inhibitors prior to ischemic insult provides significant reductions in neuronal cell death as compared to untreated controls (Eliasson et al., 1997; Endres et al., 1997; Kabra et al., 2004; Abdelkarim et al., 2001; Iwashita et al., 2004), while post-treatment with PARP-1 inhibitors also provide significant reductions in ischemic damage (Zhang et al., 1994; Takahashi et al., 1999). Thus, inhibition of PARP-1 provides neuroprotective effects when administered before, during, and after ischemic insult. In each case, PAR synthesis is ablated, which indicates that the effects are a result of the inhibition of PARP-1 activity.

PARP inhibition provides long-term neuroprotective effects as measured by decreased neuronal injury and improved motor function tests (Ding et al., 2001; Goto et al., 2002). In addition, PARP-1 deletion or pharmacologic inhibition decreases neuronal injury caused by proinflammatory mediators known to contribute to the secondary neuronal injury following stroke such as neutrophils and glia (Ullrich et al., 2001; Couturier et al., 2003; Ha, 2004). Taken together, the studies provide compelling evidence demonstrating that targeting poly(ADP-ribosyl)ation via PARP-1 may provide the most efficacious treatment of stroke to date. Therefore, poly(ADP-ribosyl)ation is a feasible drug target for the treatment of stroke.

3 PARP-1 and Apoptosis-Inducing Factor

3.1 Apoptosis-Inducing Factor

3.1.1 Features

AIF is a 67-kDa death-promoting protein discovered in 1999 that resides in the mitochondrial intermembrane space (Susin et al., 1999). Following appropriate death stimuli, such as treatment with the DNA alkylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), hydrogen peroxide, or NMDA, AIF translocates from the mitochondria to the nucleus, where it induces DNA fragmentation and triggers a caspase-independent type of cell death (Ye et al., 2002; Yu et al., 2002, 2003). The nuclear target is a yet-to-be-identified endonuclease, although it is possible that AIF may have other interactions that lead to the wide-scale DNA fragmentation. Bcl-2 and a member of the heat-shock protein 70 family of proteins can prevent AIF release from the mitochondria, but the mechanisms are unknown (Ruchalski et al., 2003). Disruption of the AIF gene in mice results in embryonic lethality, which suggests a role for AIF in embryonic development (Klein et al., 2002), although the exact biological role of AIF is not known. Currently, there are no known pharmacologic inhibitors of AIF, which further hinders discovery of its normal and pathologic functions.

3.1.2 Structural Biology

AIF is a 67-kDa protein, but purification of a 57-kDa form suggests some type of proteolytic processing of the full-length form (Susin et al., 1999). AIF contains a 100 amino acid N-terminal domain containing the mitochondrial localization sequence (MLS), a 27-amino acid spacer sequence, and a C-terminal flavin adenine dinucleotide (FAD)-binding domain. This domain contains oxidoreductase activities that are not required for its death-inducing capability. AIF shares homology with oxidoreductases in vertebrates, nonvertebrates, plants, and bacteria (Susin et al., 1999), while the recently solved crystal structure of murine AIF revealed that the active site structure suggests that AIF functions as an electron transferase similar to that of the bacterial ferredoxin reductases (Mate et al., 2002). This indicates that it may contribute to some degree in the electron transport chain as its normal biological function while residing in the mitochondria.

3.2 AIF-Induced Cell Death

The mitochondrion, known for the longest time as the energy producing organelle of the cell, can serve as a mediator of cell death. This cell death can involve the Bcl-2/Bax family of proteins and caspases (caspase-dependent) or it can occur independent of these proteins (caspase-independent). On its discovery, AIF was shown to translocate even in the presence of the caspase inhibitor Z-VAD-fmk, suggesting that it mediates a caspase-independent form of cell death (Susin et al., 1999). This type of cell death is well characterized in neurons (Yu et al., 2002) (see later), and is reported in other cell types (Gallego et al., 2004). However, other studies show that release of AIF (Arnoult et al., 2002; Cregan et al., 2002) or the *Caenorhabditis elegans* AIF homolog WAH-1 (Wang et al., 2002), occurs in a caspase-dependent manner. While AIF does mediate cell death that is caspase-independent, the cross talk between AIF and the caspase pathway is complex. Studies have shown AIF release following activation of caspase-2 (Robertson et al., 2004), or caspase-induced release of AIF from isolated mitochondria (Daugas et al., 2000). Therefore, it seems that in certain instances of caspase activation, AIF can be released secondary to this activation. Thus, AIF has a role in both caspase-dependent and caspase-independent cell death (Cregan et al., 2004).

Another controversial topic is the characterization of the caspase-independent cell death triggered by AIF in glutamate toxicity. Depending on the conditions and neuronal cells utilized, excitotoxic neuronal cell death has been described as necrotic or apoptotic (Nicotera et al., 1997). Necrosis has been described as a rapid, disorganized disintegration of membranes and organelles, while apoptosis is known as a controlled cell death characterized by chromatin condensation, cell shrinkage, and membrane blebbing with no disintegration of organelles (Kerr et al., 1972). PARP-1-dependent neuronal cell death, recently shown to be mediated by AIF (see later), exhibits features common to both necrosis and apoptosis.

3.3 Mediation of PARP-1-Dependent Cell Death by AIF

3.3.1 AIF in PARP-1-Dependent Cell Death

Recent studies indicate that the cell death pathway initiated by PARP-1 activation is mediated by AIF. Translocation of AIF from the mitochondria to the nucleus can be triggered by poly(ADP-ribosyl)ation and AIF is a downstream effector in PARP-1-mediated cell death (Yu et al., 2002, 2003). AIF fails to translocate in PARP-1 null fibroblasts following treatment with MNNG or hydrogen peroxide. In addition, concurrent with AIF translocation in wild-type cells is nuclear condensation, phosphatidylserine exposure, and dissipation of the mitochondrial membrane potential (Yu et al., 2002). These events appear to precede cytochrome *c* release and caspase activation. Further, they are not prevented by utilizing the pan-caspase inhibitor z-VAD-fmk. Therefore, cell death mediated by the translocation of AIF is dependent on poly (ADP-ribosyl)ation, and this translocation appears to trigger a unique type of cell death that is caspase-independent. As chemical inhibitors of PARP-1 can also block AIF translocation, it is PARP-1 enzymatic

activity that controls AIF release (Yu et al., 2002). How poly(ADP-ribosyl)ation, whether through NAD^+ depletion and/or PAR or a protein modified by PAR, regulates AIF translocation or release from the mitochondria is not known.

3.3.2 AIF Mediation of PARP-1-Dependent Excitotoxicity

The ability of AIF to mediate PARP-1-dependent cell death extends to neurons. Excitotoxicity following NMDA receptor stimulation in murine cortical neurons activates PARP-1 and induces AIF translocation (Yu et al., 2002; Wang et al., 2003). Similar to other cell types, the use of caspase inhibitors does not block cell death and AIF translocation in neurons. In contrast, PARP-1-null murine cortical neurons or wild-type neurons pretreated with PARP-1 inhibitors are completely protected against NMDA-mediated excitotoxicity, with no AIF translocation or nuclear condensation observed (Yu et al., 2002). Taken together, the studies indicate that excitotoxic neuronal injury via NMDA receptor stimulation causes the activation of PARP-1, the subsequent mitochondrial release and nuclear translocation of AIF, and neuronal cell death. The ability of poly(ADP-ribosyl)ation to regulate AIF translocation following different cytotoxic stimuli and in different cell types demonstrates the ability of PAR to act as an upstream signal following different types of genotoxic insults that determines the fate of many cell types, including postmitotic neurons in the CNS.

3.3.3 AIF in Stroke

The discovery of AIF in 1999 and the subsequent discovery that its translocation is dependent on the activation of PARP-1 have led to the further investigation of AIF as a mediator of neuronal injury following ischemia (*Table 2-1*). AIF is shown to translocate to the nucleus at 6 h following NMDA treatment in mouse cortical neurons (Yu et al., 2002) and as early as 4 h following OGD in rat cortical neurons (Plesnila et al., 2004). AIF translocates in vivo following MCAO in mice (Plesnila et al., 2004) and rats (Komjati et al.,

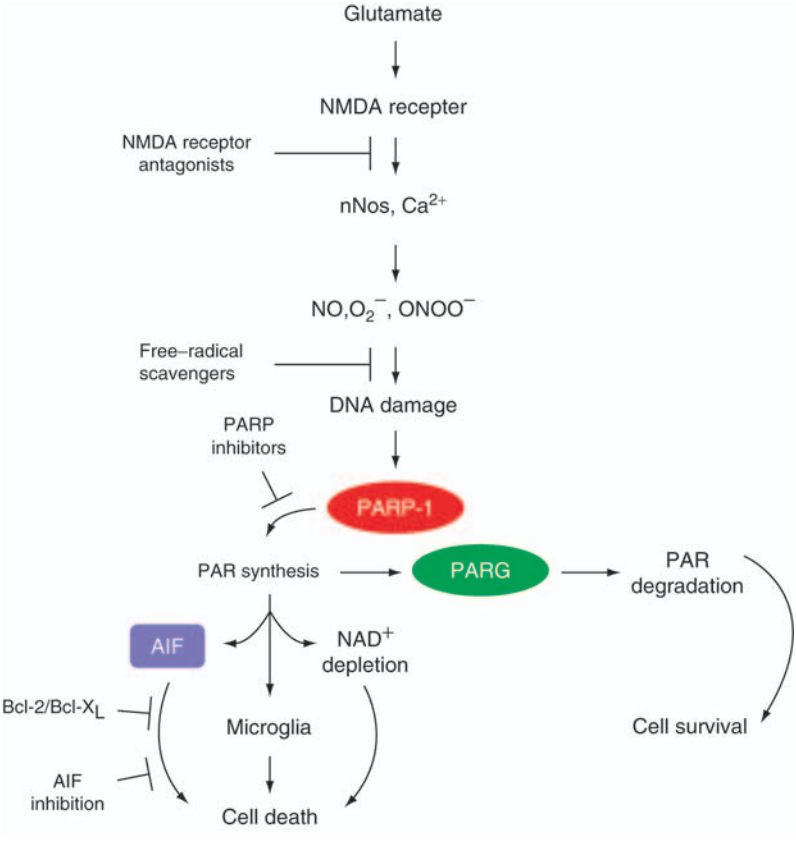
Table 2-1
Studies of AIF in experimental models of stroke

Experimental model	Insult	AIF observation	References
Mouse cortical neurons	NMDA	Translocation dependent on PARP-1 activation	Yu et al. (2002)
Rat hippocampal neurons	OGD	Translocation blocked by transient overexpression of Bcl-x_L	Cao et al. (2003)
Rat	H-I	Immediate translocation in vivo	(Zhu et al., 2003)
Rat cortical neurons	OGD	Translocation after 4 and 8 h in vitro	Plesnila et al. (2004)
Mouse	MCAO	Translocation after 1 h in vivo	
Rat	MCAO	Translocation in striatum; blocked by PARP-1 inhibition	Komjati et al. (2004)
Rat	MCAO	Translocation after 8, 24, and 48 h; blocked by Bcl-2	Zhao et al. (2004)
Rat	H-I	Translocation after 3 h	Zhu et al. (2004)
Mouse cortical neurons	NMDA	Translocation prior to cyt *c* release and caspase-3 activation	Wang et al. (In press)
Mouse	NMDA	Translocation 6 h after intrastriatal injection; no cyt *c* release	

AIF, apoptosis-inducing factor; NMDA, *N*-methyl-D-aspartate; PARP-1, poly(ADP-ribose) polymerase-1; OGD, oxygen-glucose deprivation; H-I, hypoxia-ischemia; MCAO, middle cerebral artery occlusion; cyt *c*, cytochrome *c*

Figure 2-2

The role of poly(ADP-ribosyl)ation in the events following stroke. Excitotoxicity, a major trigger for secondary neuronal injury following stroke, leads to the generation of DNA-damaging reactive oxygen species (ROS). This activates the nuclear enzyme polyadenosine diphosphoribose polymerase-1 (PARP-1) to catalyze the synthesis of poly(ADP-ribose) (PAR) polymers using NAD^+ as substrate. High levels of PAR production following stroke leads to cell death through: (1) the consumption of cellular energy stores by NAD^+ depletion; (2) the activation of microglia, which release proinflammatory cytokines at the site of injury; and (3) the translocation of AIF to the nucleus, which leads to DNA fragmentation and nuclear condensation. All three are initiated and dependent on PARP-1 activation. Accordingly, PARP-1 inhibition provides the highest level of neuroprotection in animal models of stroke. The role of PARG in stroke is unknown, but recent studies suggest that PARG has a protective role following DNA damage, and it may therefore promote cell survival after neuronal injury. NMDA receptor antagonists, free-radical scavengers, PARP inhibitors, and AIF inhibitors are all potential pharmacologic interventions that may reduce the amount of neuronal cell death following stroke, but the greatest amount of neuroprotection to date has been demonstrated through PARP-1 inhibition. Currently, there are no known pharmacologic inhibitors of AIF but the antiapoptotic proteins Bcl-2/Bcl-x_L can prevent its release from the mitochondria and thus promote cell survival



2004; Zhao et al., 2004). Hypoxia-ischemia (H-I), a different model of stroke, also produces AIF translocation following injury (Zhu et al., 2003; 2004). AIF is released from the mitochondria prior to cytochrome *c* (cyt *c*) release and caspase activation in vitro and in vivo (Yu et al., 2002; Wang et al., 2004), further supporting the caspase-independent death pathway mediated by this death protein. The feasibility of targeting AIF for stroke is evident from the neuroprotection observed by blocking AIF translocation

using PARP-1 inhibition (Yu et al., 2002; Komjati et al., 2004; Wang et al., In press), polyclonal AIF antibody (Yu et al., 2002), or overexpression of Bcl-2/Bcl-x_L (Cao et al., 2003; Zhao et al., 2004). Taken together, the studies provide compelling evidence demonstrating that targeting AIF (and poly(ADP-ribosyl)ation via PARP-1) may provide a novel treatment of stroke.

3.3.4 PARG and Neuronal Cell Survival

The putative importance of PARG in poly(ADP-ribosyl)ation suggests that it has a central role in the events following stroke and may have a role in neuronal death (*Figure 2-2*). Prior studies attempting to determine the role of PARG following ischemia cannot be justifiably interpreted due to the use of PARG inhibitors that were recently shown to be nonspecific, nonselective, and contain free-radical scavenging capabilities (Falsig et al., 2004). The loss of PARG in *Drosophila melanogaster* leads to lethality in larval stage, but PARG null mutants grown to adulthood by increasing the developmental temperature exhibit progressive neurodegeneration with reduced locomotor activity (Hanai et al., 2003). Further, they exhibit approximately a 50% decrease in lifespan as compared to wild-type animals and an extensive accumulation of PAR polymer, especially in the developing CNS. Therefore, the proper regulation of poly(ADP-ribosyl)ation may be required for proper neuronal cell function and survival in *Drosophila*. In mammals, hypomorph knockout of PARG increases the susceptibility to genotoxic stress. However, it remains to be seen what role PARG has in animal models of stroke. On the basis of current literature, one could speculate that decreasing PARG levels or activity will increase stroke volume and vice versa.

Another recent study hints at a possible role of PARG in the cellular response to DNA damage. In this study, the 110-kDa full-length isoform of PARG was reduced and the mice are viable and fertile, but they were shown to be hypersensitive to DNA damage due to a reduction in the life span following treatment with DNA-alkylating agents (Cortes et al., 2004). This suggests that PARG has a protective role in the cellular response to DNA damage. However, the aforementioned study did not completely deplete PARG levels, and PAR levels were not completely characterized, since the accumulation of PAR would be expected in mice containing lower amounts of PARG. However, this data suggests PARG may have a protective role and perhaps may protect neurons, while PARG inhibition may contribute to neuronal cell death following ischemia. In addition, it is possible that PARG may play a role in AIF function since the translocation of AIF is dependent on the ability of PARP-1 to synthesize PAR polymer. Therefore, PARP-1 may be a mediator of neuronal cell death, but PARG may be mediator of neuronal cell survival.

4 Conclusions

In summary, poly(ADP-ribosyl)ation and AIF play important roles in the life and death of neurons, and this role can be applied not only to stroke but also a broad set of clinical applications. In addition, it is now evident that PAR can be viewed as a signaling biomolecule and not just a posttranslational chemical modification of proteins. Poly(ADP-ribosyl)ation is a potential energetically expensive pathway, so it seems logical that PAR has an important cell signaling role that determines the fate of neurons via AIF. Previous studies have determined the roles of NMDA receptors and NO in the pathogenesis of neuronal injury following stroke, and they in turn elevated these key players into targets for the treatment of ischemia. Now poly(ADP-ribosyl)ation (through PARP-1) and AIF are emerging as downstream participants in this pathway and each is emerging as a therapeutic target in stroke, as well as Parkinson's disease, myocardial infarction, inflammation, and reperfusion injury. Many details remain unknown, such as the mechanism of poly(ADP-ribosyl)ation mediation of AIF function, the signal or signal transduction that causes AIF release and how it reaches the mitochondria, the nuclear target(s) of AIF, the role of AIF in mitochondria, and the efficacy of AIF inhibition in the treatment of stroke. PARP-1 and AIF studies in the future should allow insight to be gained into the complex signaling that occurs following stroke. As a consequence, this better understanding of the events following ischemia should allow improved treatment for this widespread and debilitating condition.

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3 iNOS and COX-2 in Ischemic Stroke

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Abstract: Stroke remains a leading cause for mortality and morbidity in the world despite the intense effort in research and development for remedies. Although the detailed mechanisms leading to brain tissue damage after ischemic stroke are not totally known, it is clear that inflammation plays a key role in the development of such damage. Research over the years indicates that the major players in the postischemic inflammation are inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). iNOS is expressed de novo after ischemic stroke and participates in the late phase of tissue damage. Inhibition of iNOS activity or iNOS gene deletion in rodent models of ischemic stroke provides neuroprotection. COX-2 is induced in response to ischemic and neuroexcitotoxic injuries. COX-2 inhibition with specific inhibitors reduces brain injury caused by focal ischemia. COX-2 gene deletion provides similar protection. Although iNOS and COX-2 are deleterious after stroke, recent evidence indicates that these enzymes can also be beneficial. Therefore, therapeutic approaches based on iNOS and COX-2 inhibition have to target the neurotoxic effects, while sparing the beneficial effects of the enzymes.

List of Abbreviations: CBF, cerebral blood flow; C/EBP, CCAAT/Enhancer Binding Protein; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; COX-3, cyclooxygenase-3; HMG CoA, (3-hydroxy-3-methyl-glutary1)-coenzyme A; JNK, c-jun N-terminal kinase; IPC, ischemic preconditioning; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; MCA, middle cerebral artery; NF-IL6, nuclear factor interleukin-6; NMDA, N-methyl-D-aspartic acid; NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible NOS; nNOS, neuronal NOS; eNOS, endothelial NOS; PARP, poly(ADP-ribose) polymerase; PGH2, prostaglandin H2; PGG2, prostaglandin G2; PGE2, prostaglandin E2; PGI2, prostaglandin I2; PGD2, prostaglandin D2; PGF2α, prostaglandin F2α; RNA, ribonucleic acid; ROS, reactive oxygen species; SOD, superoxide dismutase; tPA, tissue plasminogen activator; UTR, untranslated region

1 Introduction

Stroke is a leading cause of death and disability in developed countries for which there are currently few therapeutic options available. Although thrombolysis with tissue plasminogen activator (tPA) has been shown to be beneficial, the risk of hemorrhage when tPA is used beyond the therapeutic window of 3 h restricts the number of patients who can benefit from it (tPA Study Group. 1995). Therefore, there is an urgent need for developing mechanistically based therapeutic approaches that can be used either in conjunction with thrombolysis or as the main therapy in cases where thrombolytics cannot be used.

Focal cerebral ischemia is caused by reduced blood flow in the territory of affected cerebral arteries. Tissue damage after focal cerebral ischemia is the result of the concerted action of several events in a stereotyped time sequence (Dirnagl et al., 1999; Lo et al., 2003). One of the major early events is the activation of NMDA type of glutamate receptor (Lee et al., 2000). NMDA receptor activation leads to calcium overload in the cell, which activates cellular-dependent enzymes and initiates changes in gene expression that trigger secondary cellular processes in the ischemic territory (Arundine and Tymianski, 2003; Lo et al., 2003). Among these processes, postischemic inflammation and apoptosis are two major contributors to the late stages of the tissue damage (Dirnagl et al., 1999; Iadecola et al., 2004). Postischemic inflammation begins with the upregulation of proinflammatory cytokines in the injured area that leads to the adherence of circulating white cells to cerebral endothelial cells and invasion of the brain parenchyma. At the same time, astrocyte and microglia become activated, and macrophages accumulate in the injured areas (Clark et al., 1993). These inflammatory processes contribute to the development of brain damage (Dirnagl et al., 1999). However, the mechanisms by which these inflammatory cells exert their deleterious effects are not well understood. Evidence accumulated over the past several years suggests that nitric oxide (NO) and cyclooxygenases (COX) are involved in the mechanisms by which postischemic inflammation contributes to ischemic brain injury (Dirnagl et al., 1999). In this chapter, the evidence that inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are involved in ischemic brain injury is reviewed and their potential therapeutic significance discussed.

2 iNOS

NO is a free radical that acts either as a signaling molecule or as a neurotoxin. NO is the product of the enzyme NO synthase (NOS) through oxidation of the guanidino nitrogen of L-arginine (Griffith and Stuehr, 1995). Three isoforms of NOS have been characterized in the brain: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible or immunological NOS (iNOS). nNOS is localized in a selected group of neurons, whereas eNOS is found mainly in endothelial cells and in some neurons as well (Dinerman et al., 1994; Garthwaite and Boulton, 1995). eNOS and nNOS are expressed constitutively and their activity is regulated by changes in intracellular calcium concentration through interaction with calmodulin (Garthwaite and Boulton, 1995). Therefore, it is believed that NO production by nNOS and eNOS occurs in small bursts when cellular calcium concentration increases. In contrast, iNOS is not normally expressed in most cells but it is induced in pathological conditions, typically those associated with inflammation (Nathan, 1997). Unlike nNOS and eNOS, iNOS activity is not regulated by intracellular calcium. Once activated, iNOS produces NO continuously and in large amounts (Vodovotz et al., 1994). Therefore, iNOS expression and NO production are thought to contribute to the deleterious effects of inflammation (Gross and Wolin, 1995).

2.1 Nitric Oxide and Ischemic Brain Injury

The role of NO has been studied most extensively in rodent models of cerebral ischemia produced by occlusion of the middle cerebral artery (MCA). NO has been found to be involved in ischemic brain injury and can have both beneficial and detrimental effects, depending on the cellular compartment in which NO is generated, the cell type producing NO, and on the stage of evolution of ischemic brain injury (see Iadecola, 1997; Samdani et al., 1997 for a review). Immediately after induction of ischemia, the vasodilator effect of NO, produced mainly by eNOS, protects the brain by limiting the degree of flow reduction produced by the arterial occlusion (Huang et al., 1996). Further, evidence for a protective role of eNOS is provided by the observation that the reduction in ischemic injury by HMG-CoA reductase inhibitors, statins, is mediated by upregulation of eNOS (Endres et al., 2004). However, as ischemia develops, NO produced by nNOS, and later by iNOS, contributes to the evolution of the brain injury (Iadecola, 1997).

2.2 iNOS and Ischemic Brain Injury

As mentioned earlier, focal cerebral ischemia is associated with a marked inflammatory reaction that contributes to the evolution of the tissue injury. The timing of the iNOS expression after ischemic stroke coincides with such inflammatory reaction. In rodents, iNOS message, protein, and enzymatic activity are expressed in the postischemic brain and peak 12–48 h following permanent or transient MCA occlusion (Iadecola et al., 1995a, b, 1996; Grandati et al., 1997). The expression after ischemia is localized in inflammatory cells infiltrating the injured brain and in cerebral blood vessels. Immunocytochemical staining of the injured brain in patients who died within 24 h after a major stroke detected iNOS expression in neutrophils and vascular cells (Forster et al., 1999). In addition, cells containing iNOS immunoreactivity were also positive for nitrotyrosine, a marker of NO-derived peroxynitrite (Crow and Beckman, 1995), suggesting that iNOS is catalytically active also in the postischemic human brain.

The role of iNOS in ischemic stroke has been studied pharmacologically using systemic administration of relatively selective iNOS inhibitors, such as aminoguanidine or 1400GW (Iadecola et al., 1995c; Parmentier et al., 1999), or using iNOS-knockout mice (Iadecola et al., 1997). Because iNOS is expressed many hours after induction of ischemia, the inhibitors can be applied 12–24 h after MCA occlusion, a time period when iNOS is present. iNOS inhibitors were able to reduce infarct volume by 30%–40% when administrated within this time window (Iadecola et al., 1995c, 1996; Parmentier et al., 1999). Importantly, the reduction in tissue damage was associated with an improvement of the neurological deficits produced by the infarct (Nagayama et al., 1998). These findings suggest that iNOS inhibition can protect the brain

with a therapeutic window wider than that of other neuroprotective agents. The protection is not due to the preservation of postischemic blood flow because aminoguanidine did not influence cerebral blood flow (CBF) (Iadecola et al., 1995c). To demonstrate further that iNOS was involved in ischemic brain injury, mice lacking the iNOS gene were used (MacMicking et al., 1995). As expected, iNOS-null mice do not express iNOS in the brain following MCA occlusion and have smaller infarcts (–30%) and better neurological outcome than wild-type littermates (Iadecola et al., 1997). Consistent with a gene-dosing effect of iNOS deletion, it was found that the reduction in infarct volume was greater in homozygous than in heterozygous iNOS mice (Zhao et al., 2000). The protection was not the result of cerebrovascular effects of iNOS deletion, because there was no difference in the reduction in CBF produced by MCA occlusion between iNOS-null mice and controls (Iadecola et al., 1997). Furthermore, the protection could not be attributed to effects of iNOS-knockout on the cellular reaction that occurs after ischemia, because the degree of neutrophilic infiltration and astrocytic activation was comparable in iNOS-null mice and controls (Iadecola et al., 1997).

Additional evidence for a role of iNOS in ischemic brain injury has been provided by studies in which iNOS expression was downregulated using an antisense approach. Thus, administration of antisense, but not its scrambled controls, reduced stroke volume by 30% in a rat model of focal cerebral ischemia (Parmentier-Batteur et al., 2001).

What are the mechanisms by which NO produced by iNOS kills neurons in ischemic conditions? It seems that multiple factors are involved. Peroxynitrite, the product of the reaction of NO with the free radical superoxide, has deleterious effect on DNA, structural proteins, and enzymes (Love, 1999). Another mechanism involves competition of NO with oxygen for the enzyme cytochrome *c* oxidase. In neuron-glial cultures, NO release results in inhibition of cytochrome *c* oxidase through competition with O_2 leading to ATP depletion, glutamate release, and delayed activation of NMDA receptors (Bal-Price and Brown, 2001; Mander and Brown, 2004; Mander et al., 2005).

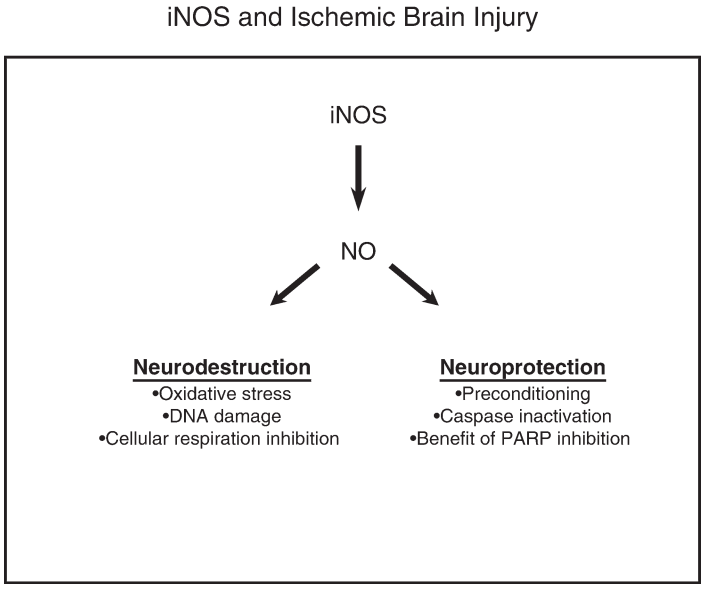
2.3 Beneficial Role of iNOS in Cerebral Ischemia

The earlier discussion illustrates the deleterious effect of iNOS expression and NO production in cerebral ischemia. However, recent evidence suggests that iNOS also has beneficial roles in ischemic brain injury as illustrated in *Figure 3-1*. For example, iNOS is needed for the neuroprotection exerted by poly(ADP-ribose) polymerase (PARP) inhibitors (Park et al., 2004). PARP is a nuclear protein that participates in DNA base-excision repair in response to DNA damage. PARP activation is involved in the mechanisms of ischemic neuronal death (Virag and Szabo, 2002). Pharmacological inhibition of PARP attenuates the brain injury produced by occlusion of the MCA in rodents (Takahashi et al., 1997; Park et al., 2004). Interestingly, the neuroprotective effect of PARP inhibition is abolished if iNOS is inhibited (Park et al., 2004). Furthermore, PARP inhibition in iNOS-null mice subjected to MCA occlusion does not produce neuroprotection (Park et al., 2004). These results suggest that iNOS is required to achieve the neuroprotection afforded by PARP inhibition, although the mechanisms are still not clear.

Another example of the protective effect of iNOS is that iNOS contributes to the establishment of ischemic preconditioning (IPC). IPC is a phenomenon in which a brief episode of ischemia can lead to remarkable protection against a subsequent lethal ischemic insult. IPC can be established in different organs such as heart, kidney, and brain. For example, it has been shown that treatment with iNOS inhibitors or using iNOS-knockout mice mitigates the protection afforded by short period of IPC in heart and kidney (Dawn and Bolli, 2002; Park et al., 2003). In the brain, treatment with the iNOS inhibitor aminoguanidine (400 mg/kg) abolishes the IPC-induced protection (Cho, in press). Furthermore, IPC fails to induce cerebral ischemic tolerance in iNOS-null mice. In wild-type mice, IPC increases the resistance to Ca^{2+}-mediated depolarization in isolated brain mitochondria. However, in iNOS-null mice, IPC fails to induce such resistance (Cho, in press). These data suggest that iNOS is required for the increased resistance of mitochondria to injury that underlies the expression of IPC. However, the molecular mechanisms of the protective effect of NO have not been established.

NO is able to react with the thiol group (SH) on cysteine residues in polypeptides, resulting in protein nitrosylation (Stamler, 1994). *S*-Nitrosylation on the cysteine residue at the catalytic active site of caspases

Figure 3-1
Effect of iNOS-produced NO in ischemic brain injury



renders the protease inactive (Mannick et al., 2001). Such NO-induced caspase inhibition has been shown to inhibit apoptosis in nonneuronal systems (Kim et al., 1997; Mannick et al., 1997; Ceneviva et al., 1998). In primary neuronal cultures, NO donor reduces caspase-3 and -9 activities and prevents the appearance of the apoptotic morphology induced by staurosporine and camptothecin (Zhou et al., 2005). Furthermore, brain slices from iNOS-null mice exposed to staurosporine have increased caspase-3 activity compared with wild-type controls (Zhou et al., 2005). These results suggest that NO and iNOS are capable of modulating caspase activity and may influence cell fate. However, it remains unclear how these effects of NO on caspases are involved in the role of NO in cerebral ischemia.

2.4 iNOS as a Therapeutic Target for Stroke

Collectively, the findings reviewed earlier provide strong evidence for a major role of NO produced by iNOS in cerebral ischemic injury. Furthermore, the fact that iNOS is expressed also in the human brain after ischemia strengthens the argument that iNOS is a valuable therapeutic target in human stroke. The extended therapeutic window of iNOS inhibitors (12–24 h) underscores the value of iNOS inhibition as a therapeutic target. This is because iNOS inhibition could be used to treat stroke patients who do not qualify for treatment with other modalities, such as thrombolysis or glutamate receptor inhibition, which are effective only in the early stages of the damage (Dirnagl et al., 1999). Therefore, inhibition of iNOS expression or activity would be a valuable therapeutic strategy to selectively target the delayed phase of the damage. However, the finding that iNOS can also be beneficial in ischemic preconditioning raises the possibilities that NO derived from iNOS can also be protective. Further studies are needed to resolve this issue.

3 Cyclooxygenase-2

COX-2 is a rate-limiting enzyme in the synthesis of prostanoids that has recently emerged as an important factor in normal and abnormal brain function. Although COX-2 has been implicated in synaptic plasticity

and neurovascular coupling (Kaufmann et al., 1996; Niwa et al., 2000; Chen et al., 2002), it has also been shown to participate in the mechanisms of neuronal death in several neurological diseases, including stroke (Hurley et al., 2002). In ischemic brain injury, COX-2 participates both in the initiation of the damage and in its progression. In this section, we examine the evidence supporting a role of COX-2 in ischemic brain injury and review recent data on the COX-2 reaction products mediating these deleterious effects.

3.1 COX Isoforms and Enzymatic Products

Cyclooxygenases are the enzymes that catalyze the reaction which converts arachidonic acid into prostaglandin H2 (PGH2) (Smith and Song, 2002) in a two-step reaction: an oxidation step in which arachidonic acid is converted into PGG2 followed by a reduction step in which PGG2 is converted to PGH2 (Garavito and Mulichak, 2003). The peroxidase step leads to the formation of equal amount of PGH2 and superoxide (Smith and Song, 2002). Cell-specific synthase and isomerase convert PGH2 into five primary prostanoids (PGE2, PGI2, PGD2, PGF2α, and thromboxane A2) (Breyer et al., 2001). Three isoforms of COX have been characterized: COX-1, COX-2, and COX-3, a recently identified COX-1 splice variant (Chandrasekharan et al., 2002). COX-1 is expressed constitutively in nearly all cell types and is involved in normal cellular function (Cohn et al., 1997; Hurley et al., 2002; Garavito and Mulichak, 2003). COX-3 is enriched in the brain and heart, and aside from a purported role in the regulation of fever and pain, its biological functions have not been elucidated (Chandrasekharan et al., 2002).

3.2 Regulation of COX-2 Expression

In the normal brain, COX-2 is constitutively expressed in a select population of glutamatergic neurons in cortex, hippocampus, hypothalamus, and spinal cord (Breder et al., 1995; Kaufmann et al., 1996; Wang et al., 2004). COX-2 has been suggested to play a role in synaptic plasticity and neurovascular regulation (Kaufmann et al., 1996; Niwa et al., 2000). However, the most striking feature of COX-2 in the brain is that this enzyme is markedly upregulated by inflammatory stimuli and other forms of cellular stress (Hurley et al., 2002). For example, cerebral ischemia leads to marked upregulation of COX-2. In rodents, as in human and nonhuman primates, COX-2 message, protein, and enzymatic activity increase markedly in the ischemic brain after occlusion of the MCA (Collaco-Moraes et al., 1996; Nogawa et al., 1997; Sairanen et al., 1998; Iadecola et al., 1999; Yokota et al., 2003). The stimulatory signals likely to induce COX-2 expression during brain ischemia include excitotoxic amino acids (Miettinen et al., 1997; Manabe et al., 2004), hypoxia (Lukiw et al., 2003), and proinflammatory cytokines (Licinio and Wong, 1997).

COX-2 gene expression is regulated at both transcriptional and posttranscriptional levels. The transcriptional regulation was mostly studied in macrophage cell lines (Wadleigh et al., 2000; Caivano et al., 2001). Lipopolysacchride (LPS)-induced COX-2 expression in RAW 264.7 macrophages requires three major *cis*-regulatory elements in the promoter region of the COX-2 gene (Wadleigh et al., 2000). In addition, the binding of C/EBP transcription factors to the nuclear factor interleukin-6 (NF-IL6) element is also essential for inducible COX-2 transcription in macrophages (Caivano et al., 2001). Another way of regulation for COX-2 expression is posttranscriptional regulation through RNA destabilization. A common feature of mRNA from many unstable immediate early genes is the presence of AU-rich segments within the 3′-untranslated region (3′-UTR) (Chen and Shyu, 1994). The COX-2 gene contains 23 copies of such AU-rich elements in its 3′-UTR. The stability of COX-2 mRNA is regulated by the p38/MAPK-signaling cascade, and MAP kinase inhibition results in destabilization of COX-2 mRNA (Lasa et al., 2000). In the brain, it is not clear whether similar transcriptional and nontranscriptional mechanisms are utilized to regulate COX-2 expression upon ischemic injury. In models of neurodegeneration, it has been shown recently that c-Jun phosphorylation by c-Jun N-terminal kinase (JNK) is a critical step for COX-2 expression

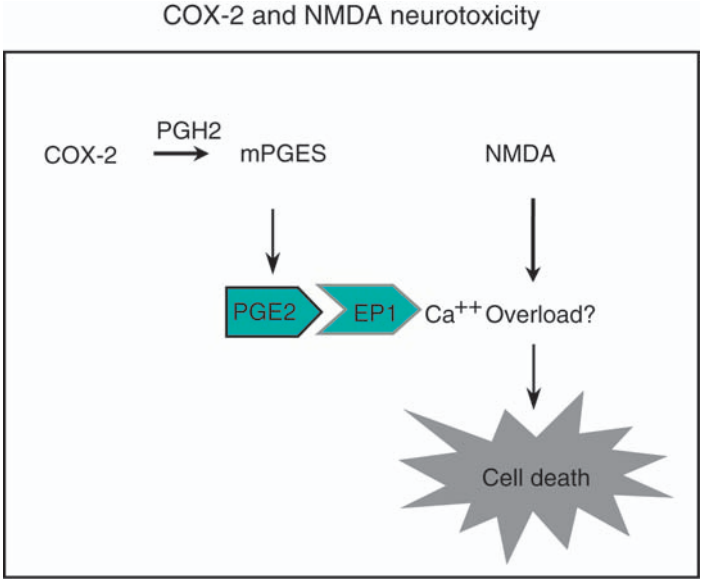
(Hunot et al., 2004). It remains to be established whether this mechanism is also involved in postischemic COX-2 expression.

3.3 COX-2 and Ischemic Brain Injury

The role of COX-2 in brain damage produced by focal cerebral ischemia was studied mostly in rodents using pharmacological inhibitors and COX-2-knockout mice. Administration of relatively selective COX-2 inhibitors attenuates COX-2 enzymatic activity and reduces ischemic injury, while COX-2-knockout mice are relatively protected from focal or global cerebral ischemia (Nogawa et al., 1997; Nakayama et al., 1998; Govoni et al., 2001; Iadecola et al., 2001; Lapchak et al., 2001; Sasaki et al., 2004). On the other hand, neuronal COX-2 overexpression in transgenic animals exacerbates ischemic injury (Dore et al., 2003). Studies using COX-2-knockout mice demonstrated that COX-2 is involved in both early and late pathogenic events in the postischemic brain. When brain injury was examined 24 h after MCA occlusion, COX-2-null mice were found to have smaller infarcts at this time point (Iadecola et al., 2001). This is in clear contrast to iNOS-knockout mice in which there is no protection 24 h after MCA occlusion since the iNOS activity participates mostly in the late phase of postischemic damage (Iadecola et al., 1997). The fact that COX-2-null mice are protected at 24 h suggests a role of COX-2 in the early phase of cerebral ischemia. On the other hand, inhibition of COX-2 activity with NS398 24 h after MCA occlusion reduces infarct volume as well (Sugimoto and Iadecola, 2003), suggesting a role of COX-2 also in the late phase of the damage.

In agreement with its role in the early stages of cerebral ischemia, COX-2 is involved in the neurotoxicity mediated by activation of NMDA receptors (see *Figure 3-2* for illustration). The COX-2 inhibitor NS398 attenuates the injury produced by NMDA both in vitro and in vivo (Hewett et al., 2000; Iadecola et al., 2001;

Figure 3-2
COX-2 activation contributes to NMDA-induced cell death



Mirjany et al., 2002; McCullough et al., 2004). Furthermore, COX-2-null mice have reduced injury following activation of glutamate receptor (Iadecola et al., 2001), while COX-2 transgenic mice are more susceptible to such injury (Kelley et al., 1999). The role of COX-2 in the inflammatory component of cerebral ischemia

is demonstrated in the experiment in which inhibition of COX-2 attenuates the neuronal damage produced by the proinflammatory agent lipopolysaccharide in neuronal cultures (Araki et al., 2001).

3.4 COX-2-Derived ROS Do Not Play a Major Role in Neurotoxicity

Which COX-2 product mediates the neurotoxic effect in ischemic tissue damage? Recent data have begun to shed light on this question (Carlson, 2003; Manabe et al., 2004). As discussed earlier, COX-2 reaction produces reactive oxygen species (ROS) and prostanoids. It is well established that ROS is involved in the mechanisms of ischemic brain injury (Traystman et al., 1991; Chan, 2001; Sugawara and Chan, 2003). There is marked ROS production and oxidative stress associated with focal cerebral ischemia (Dugan et al., 1995; Morimoto et al., 1996; Piantadosi and Zhang, 1996; Peters et al., 1998; Mackensen et al., 2001; Kim et al., 2002). Consequently, antioxidants and free-radical scavengers attenuate the deleterious effects of ROS and reduce ischemic brain injury (Liu et al., 1989; Mackensen et al., 2001). In addition, transgenic mice overexpressing the ROS-scavenging enzyme superoxide dismutase (SOD) have smaller infarcts after MCA occlusion, while mice deficient in SOD exhibit larger infarcts (Kinouchi et al., 1991; Kondo et al., 1997; Murakami et al., 1998). Taken together, these results underscore the important role of ROS in ischemic brain damage. Therefore, COX-2-derived ROS could contribute to the postischemic brain injury. To address this issue, we used an in vivo model of NMDA neurotoxicity. ROS production following NMDA injection was assessed by hydroethidine microfluorography, a method based on the ability of superoxide to convert hydroethidine to ethidium (Bindokas et al., 1996; Murakami et al., 1998; Tarpey et al., 2004). We found that, although NMDA injection into the cerebral cortex increases local ROS production, COX-2 inhibition did not attenuate such increase in ROS production (Manabe et al., 2004). Similarly, ROS production was not reduced in COX-2-null mice (Manabe et al., 2004). These data suggest that COX-2-generated ROS does not contribute to NMDA-induced oxidative stress and implicate prostanoids in the mechanisms of the damage.

3.5 COX-2-Derived Prostanoids Are Involved in COX-2-Dependent Neurotoxicity

Recent data suggest that PGE2 is the prostanoid linked to COX-2-dependent brain damage. Injection of 17-pt-E2, a PGE2 stable analog, abolished the protective effect of COX-2 inhibition in a model of NMDA-induced injury in vivo (Manabe et al., 2004). Similar results were obtained in neuronal cultures (Carlson, 2003). These in vitro and in vivo findings support the hypothesis that PGE2 is a major contributor in the COX-2-dependent component of the excitotoxic injury. However, it remains to be established whether PGE2 is the COX-2 reaction product that mediates COX-2-dependent damage in the postischemic brain.

3.6 Prostanoid Receptors

The biological effects of prostanoids are mediated through specific G protein-coupled receptors with seven transmembrane domains (Kobayashi and Narumiya, 2002; Tsuboi et al., 2002). Four different receptor subtypes exist for PGE2: EP_1, EP_2, EP_3, and EP_4, each coupling to its unique G protein and mediating different signaling pathways (Kobayashi and Narumiya, 2002; Tsuboi et al., 2002). EP_1 receptors couple through G_q and increase intracellular calcium if expressed in oocytes, while EP_2 and EP_4 receptors couple through G_s and increase intracellular cAMP (Funk et al., 1993; Kobayashi and Narumiya, 2002; Tsuboi et al., 2002). In contrast, EP_3 receptors act via G_i and inhibit the increase in cAMP (Kobayashi and Narumiya, 2002; Tsuboi et al., 2002). The opposing effects of EP receptors suggest that in neurons PGE2 can be either protective or destructive, depending on the existing milieu and conditions (Akaike et al., 1994; Cazevieille et al., 1994; Bezzi et al., 1998; Thornhill and Smith, 1998; Carlson, 2003; Takadera et al., 2004). This may explain why there are conflicting reports about the effect of PGE2 on neurons (see refs. Breyer, 2001; McCullough et al., 2004 for a discussion). EP receptors in the brain have been examined in the context of

the role of PGE2 in thermoregulation and pain perception (Ushikubi et al., 1998; Oka et al., 2003), but little is known about their cellular and subcellular localization in different brain regions. A recent study suggests hat EP_2 receptors are present in the brain and their activation is neuroprotective against injury produced by NMDA and oxygen and glucose deprivation. The beneficial effect is thought to be mediated by the associated increase in cAMP (McCullough et al., 2004). Our recent finding that the EP_2 receptor agonist butaprost reduces the brain damage produced by microinjection of NMDA into the cerebral cortex supports this conclusion (Anrather et al., 2004). The effects of other EP receptors in brain injury are largely unknown. However, it has been reported that EP_1 receptors are involved in the cytotoxicity induced by hypertension in the kidney or by histamine in the stomach (Hase et al., 2003; Suganami et al., 2003). Preliminary results suggest that EP_1 receptors are involved in the neurotoxicity produced by activation of NMDA receptors (Kawano et al., 2004) (*Figure 3-2*). However, their role in ischemic brain injury needs to be studied.

3.7 Therapeutic Potential of the COX-2 Pathway

Although COX-2 inhibitors have potential in the treatment of ischemic stroke, recent clinical trails have concluded that COX-2 inhibitors have increased the incidents of stroke and myocardial infarction (Iadecola et al., 2005). As a result of these findings, a popular COX-2 inhibitor has been withdrawn from the market. The deleterious vascular effects of COX-2 inhibition have been attributed to the reduction in COX-2-derived prostacyclin, a prostanoid with beneficial vascular effects (vasodilation, antiplatelet aggregation). Therefore, in order to take advantage of the therapeutic potential of the COX-2 pathway, therapeutics targeting the downstream effectors of COX-2 need to be developed. Preclinical studies addressing these issues are currently underway.

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4 eNOS and nNOS in Stroke

P. L. Huang

Abstract: Nitric oxide (NO) is an important biological mediator. In the brain, NO is involved in development, neuronal plasticity, neurotransmission, regulation of cerebral blood flow, and coupling of blood flow to metabolism. NO generated by the three isoforms of nitric oxide synthase (NOS), endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS), plays distinct roles. Following cerebral ischemia, NO levels rise dramatically, and NO can be either toxic or protective. The purposes of this chapter are: (1) to review the differences between NOS isoforms, (2) to describe the roles of NO in the brain, (3) to demonstrate how genetic approaches to studying NO have defined the individual roles of eNOS and nNOS in stroke, and (4) to describe next generation NOS mutant mice.

List of Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NMDA, N-methyl-D-aspartate; PET, positron emisson tomography; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; BH4, tetrahydrobiopterin; TTC, 2,3,5-triphenyltetrazolium chloride; cGMP, cyclic guanosine monophosphate; 7-NI, 7-nitroindazole

1 Introduction

Nitric oxide (NO) is a gas produced by cells as a messenger molecule that serves a variety of purposes. NO was virtually unheard of 30 years ago. The pioneering work of Furchgott (Furchgott and Zawadzki, 1980), Ignarro (Ignarro et al., 1987), Murad (Arnold et al., 1977), Snyder (Snyder and Bredt, 1991), and Moncada (Palmer et al., 1987) have been seminal contributions to our understanding of the many roles of this unique, and in many ways, atypical signaling molecule. As a gas, NO may diffuse across cell membranes, allowing it to act either in the same cell or in the neighboring cells. Unlike classical neurotransmitters, NO cannot be stored in vesicles for later release, and it does not bind to classical cell surface receptors (Boehning and Snyder, 2003). NO's half-life in vivo is extremely limited. Biological roles for NO range from neurotransmission and intercellular communication to regulation of blood flow and defense against pathogens (Bredt and Snyder, 1992). In the brain, NO is involved in development, neuronal plasticity, neurotransmission, regulation of cerebral blood flow, and coupling of blood flow to metabolism. It is now clear that NO may play both toxic and protective roles, depending on the pathophysiologic context (Beckman and Koppenol, 1996). In this chapter, we (1) review the differences between nitric oxide synthase (NOS) isoforms, (2) describe the roles of NO in the brain, (3) demonstrate how genetic approaches to studying NO, including knockout mice, have defined the individual roles of eNOS and nNOS in stroke, and (4) describe next generation NOS mutant mice and their potential uses in dissecting the multiple roles of NO in the brain.

2 Nitric Oxide Synthases

2.1 Structural Features

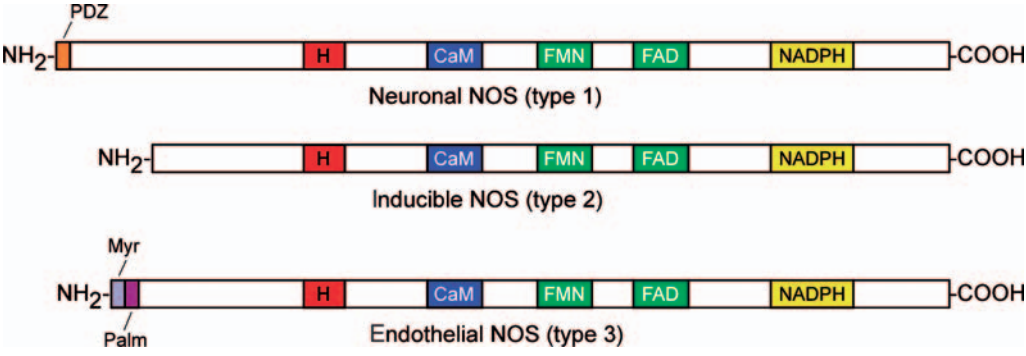
NO is produced by a family of three NOS isoforms (Alderton et al., 2001): neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). *Table 4-1* lists some of the properties of these isoforms. They all utilize the amino acid L-arginine as substrate and produce NO by a series of electron transfer reactions, resulting ultimately in the oxidation of one of the terminal guanido nitrogen atoms to generate NO. The three NOS isoforms are encoded by separate genes located on separate chromosomes.

As shown in *Figure 4-1*, the NOS isoforms share regions of homology, including binding sites for NADPH ribose and adenine, flavin adenine dinucleotide, and flavin mononucleotide. They each have unique structural features as well. nNOS has a PDZ domain (Brenman et al., 1996) not found in eNOS or iNOS. The PDZ domain is located at its N terminus and is involved in protein–protein interactions that localize nNOS to the postsynaptic membrane in neurons and to the neuromuscular junction in skeletal muscle. eNOS is also restricted in its subcellular localization (Shaul, 2002). eNOS undergoes fatty

Table 4-1
NOS isoforms

Common name Typical cell	Type I NOS (nNOS) Neurons	Type II NOS (iNOS) Macrophages	Type III NOS (eNOS) Endothelium
Other sites of expression	Smooth muscle Skeletal muscle	Smooth muscle Liver	Smooth muscle platelets
Expression pattern	Constitutive	Inducible	Constitutive
Regulation	Calcium dependent	Gene transcription	Calcium-dependent S1179 phosphorylation
Output	Moderate (nM to μM)	High (μM)	Low (pM to nM)
Intracellular location	Sarcolemma	Cytoplasm	Caveolae
Means of localization	N-terminal PDZ domain	N/A	N-terminal myristoylation

Figure 4-1
Structural features of NOS isoforms. The three isoforms share homologies in regions involved in cofactor binding (FMN, FAD, NADPH ribose and adenine), calmodulin binding (CaM), and heme binding (H). The nNOS isoform has a PDZ domain at its N terminus. The eNOS isoform undergoes myristoylation and palmitoylation at cysteine residues near its N terminus



acid modifications of palmitoylation and myristoylation at Cys2 and Cys15. These modifications are involved in the localization of eNOS to caveolae, specialized invaginations of the plasma membrane in endothelial cells.

2.2 Regulation of Activity

nNOS and eNOS are dependent on intracellular calcium for their activation, and in many cells, they are produced constitutively (Nathan and Xie, 1994; Alderton et al., 2001). Thus, they have been known as constitutive NOS isoforms. Production of NO by eNOS and nNOS is regulated mainly by controlling intracellular calcium concentrations. In contrast, iNOS is not produced constitutively, but rather its expression can be induced by activation of its transcription by stimuli such as lipopolysaccharide or tumor necrosis factor. Regulation of NO production by iNOS is through regulation of its expression.

These differences result from the different ways NOS isoforms bind to cofactors and dimerize. Dimerization is required for enzymatic activity. For nNOS and eNOS, NOS monomer proteins first bind

to the cofactors FAD and FMN. Addition of the substrate L-arginine, the cofactor tetrahydrobiopterin, and a heme group allows the NOS protein to dimerize, but the dimers are still inactive. Full activity results when intracellular calcium concentrations increase, resulting in calmodulin binding to the dimers. In contrast, the iNOS isoform binds FAD, FMN, and calcium/calmodulin even at low (resting intracellular) concentrations of calcium. Addition of L-arginine, tetrahydrobiopterin, and heme allows iNOS to dimerize and activates it. Thus, the main switch for activity for nNOS and eNOS is a transient increase in intracellular calcium concentration, whereas the main switch for iNOS is by transcription.

2.3 NOS Isoforms in the Brain

Although the three NOS isoforms differ in their tissue- and cell-specific expression patterns, all three are important in the brain. nNOS is expressed by specific populations of neurons in the cortex and striatum, the hippocampus, and the cerebellum. Although it has been estimated that 2% of neurons in the cortex and striatum produce nNOS, these neurons have widespread projections that enable NO to reach almost the entire volume of the brain (Snyder and Bredt, 1991, 1992; Snyder, 1992). eNOS is expressed in the endothelium of the cerebrovasculature, thus also allowing NO to reach the entire volume of the brain. eNOS is also expressed in populations of neurons in the hippocampus and cerebellum (O'Dell et al., 1994; Son et al., 1996). iNOS is expressed in glial cells and astrocytes as well as in circulating macrophages that may be recruited to areas of injury and inflammation. Therefore, all three NOS isoforms are expressed in the brain and may mediate effects relevant to normal brain function and response to ischemia, infarction, and inflammation.

3 Mechanisms of NO Action

3.1 cGMP-Dependent Mechanisms

One of the principal mechanisms for NO to mediate downstream biological effects is the activation of soluble guanylyl cyclase and the production of cGMP (Snyder and Bredt, 1992; Dawson et al., 1994a). This is responsible for events in the brain following N-methyl-D-aspartate receptor activation. Garthwaite first described that cultures of cerebellar cells produce cGMP in response to the excitatory amino acid neurotransmitter glutamate (Garthwaite and Balazs, 1978). This is due to the activation of nNOS by calcium influx; the NO produced then binds to the heme in soluble guanylyl cyclase, activating it, and resulting in increased production of cGMP.

In the vasculature, cGMP mediates NO-dependent relaxation of vascular smooth muscle, resulting in vasodilation. Similarly, NO produced as a neurotransmitter in the autonomic nervous system innervating the gastrointestinal tract, urinary tract, and the respiratory tract mediates smooth muscle relaxation in these tissues by increases in cGMP production. These effects are likely mediated by the phosphorylation of downstream proteins by cGMP-dependent protein kinases, including myosin light chain.

3.2 cGMP-Independent Mechanisms

There are a number of cGMP-independent effects of NO, as listed in *Table 4-2*. First, NO can S-nitrosylate proteins (Stamler, 1994), including hemoglobin, which may serve as a natural carrier (Stamler et al., 1997). Second, NO can react with superoxide to form peroxynitrite anion. Peroxynitrite in turn can react with tyrosine residues in proteins to form nitrotyrosine (Beckman and Crow, 1993; Beckman and Koppenol, 1996). Third, NO can directly inhibit mitochondrial complexes I and IV (Brown, 1995; Takehara et al., 1995; Okada et al., 1996). Finally, NO can activate the enzyme poly-ADP ribose polymerase (PARP), resulting in depletion of cellular energy stores (Dawson et al., 1994b; Endres et al., 1998b). Generally, these

Table 4-2
Mechanisms of NO action

Mechanism	NO effect	Biological effects
Production of cGMP	Activation of soluble guanylyl cyclase	Relaxation of smooth muscle
S-Nitrosylation	Formation of *S*-nitrosothiols on proteins	Altered enzymatic and biological activities
Peroxynitrite formation	Reaction with superoxide to form peroxynitrite	Oxidative damage
Activation of PARP		Depletion of energy stores
Mitochondrial inhibition	Binding to and inhibiting mitochondrial complexes I and IV	Blockade of cellular respiration

non-cGMP-dependent mechanisms underlie some of the toxicity of NO, while effects on soluble guanylate cyclase mediate many of the biological signaling roles of NO.

4 Approaches to Study NOS Isoforms

4.1 Nongenetic Approaches

4.1.1 Pharmacologic Inhibitors

There are several approaches to studying the biological roles of NO. An important approach is the use of NOS inhibitors. Analogs of L-arginine, the substrate for NOS enzymes, have been used as pharmacologic inhibitors of NOS. These include L-nitroarginine (L-NA), L-*N*-arginine methyl ester (L-NAME), L-*N*-monomethyl-arginine (L-NMMA), and other related compounds. Many of these compounds have substitutions at the guanidine nitrogen(s), rendering the molecule unable to serve as substrate for NO generation. If these compounds block a specific biological process, and if the inhibition can be overcome by L-arginine but not by D-arginine, it is strong pharmacologic evidence that NO derived from NOS may be involved in the process. There are also inhibitors of NOS enzymes that are structurally distinct from arginine analogs, e.g., 7-nitroindazole (7-NI).

4.1.2 Markers of NO Generation

A second approach is to detect markers of NO generation or downstream effects. Measurements of tissue cGMP can show increases associated with NO generation, and this can be modulated by cGMP analogs such as 8-Br cGMP. NO is converted to nitrite and nitrate, the measurement of whose levels in blood, urine, or tissues can be useful as a measure of amount of NO generated. Peroxynitrite, formed by the reaction of NO with O_2^-, catalyzes nitration of tyrosine residues in proteins. Immunoreactivity for nitrotyrosine is an indication of peroxynitrite formation (Beckman and Crow, 1993; Beckman and Koppenol, 1996). Finally, antibodies to citrulline, the other end product of NOS enzymes, can be used to measure NOS activity (Eliasson et al., 1999).

4.1.3 Limitations

Because nNOS, eNOS, and iNOS share L-arginine as substrate and similarities in enzyme catalysis mechanism, they may all be affected to some extent by pharmacologic inhibitors. Some agents display more effect on one isoform over another, either in vitro or in vivo, but lack of specificity between isoforms is a major limiting factor in the use of pharmacologic agents to distinguish between nNOS and eNOS. Similarly, measurement or modulation of cGMP levels does not distinguish between NO derived from eNOS, nNOS, or iNOS.

4.2 Targeted Disruption of NOS Genes

Genetic approaches have been useful to distinguish between the roles of individual NOS isoforms. Gene knockout of the nNOS gene, the eNOS gene, and the iNOS gene results in mice that lack each isoform. Mice lacking nNOS, eNOS, and iNOS are all viable.

The study of NOS gene knockout mice has several advantages. First, it circumvents the lack of specificity of pharmacologic NOS inhibitors. Second, it pinpoints the roles of individual NOS genes since many tissues contain all three of the major NOS isoforms. Third, it allows the study of how chronic absence of the NOS isoform affects physiology in intact animals.

Several issues may potentially confound studies using knockout animals. There may be developmental abnormalities due to the gene knockout. If one of the NOS isoforms plays a critical role in embryonic development, its absence may lead to other secondary abnormalities that are difficult to predict. There may be physiologic compensation for the absence of individual NOS genes (Irikura et al., 1995; Huang and Fishman, 1996; Ma et al., 1996a, b). Genetic background may confound the observed phenotype, particularly when the parental background strains of the knockout mice behave differently from one another (Gerlai, 2001). For this reason, most studies now use animals that have been backcrossed for ten generations to an inbred strain, e.g., the C57BL/6 strain.

Table 4-3 summarizes some of the phenotypes of the NOS knockout mice. nNOS knockout mice show pyloric stenosis, confirming the importance of nNOS to gastrointestinal motility and smooth muscle

Table 4-3
NOS knockout mice

Isoform deleted	Phenotype	Reference
nNOS	Pyloric stenosis	Huang et al. (1993)
	Normal brain development	
	Resistance to stroke	Huang et al. (1994), Panahian et al. (1996)
iNOS	Susceptibility to pathogens	MacMicking et al. (1995)
	Resistance to septic hypotension	Wei et al. (1995) Laubach et al. (1995)
	Decreased atherosclerosis	Kuhlencordt et al. (2001a)
eNOS	Hypertension	Huang et al. (1995)
	Absent EDRF activity	
	Increased vascular injury	Moroi et al. (1998)
	Increased atherosclerosis	Chen et al. (2001), Kuhlencordt et al. (2001b)

relaxation in the gut. Their brain development appears to be normal. iNOS knockout mice are susceptible to infections that require cell-mediated immunity for defense and are resistant to hypotension in sepsis. eNOS knockout mice are hypertensive and lack EDRF activity. They show an exaggerated response to vessel injury and are prone to atherosclerosis. We detail later how these and other mouse models have been used to study the roles of NO in cerebral blood flow and stroke.

5 Roles of NO in the Brain

5.1 Neuronal Plasticity

NO mediates many of the effects of glutamate neurotransmission following NMDA receptor activation. By extension, excess NO may also mediate many effects of excitotoxicity. The unusual features of NO—its ability to diffuse across cell membranes and the lack of a requirement for a specific cell-surface receptor—have led to speculation that NO serves novel signaling functions.

NO has been proposed to serve as the elusive retrograde transmitter in activity-dependent modulation of synaptic circuits (Edelman and Gally, 1992). Activity-dependent changes in circuitry are fundamentally important to higher neurologic processes such as learning and memory. In these processes, certain synapses are strengthened and others are weakened, depending on their activity or the activity of nearby neurons. The strengthening involves changes in the presynpatic neuron, but it has not been clear how the presynaptic neuron knows if its signal has been received by the postsynaptic neuron. Theoretically, a retrograde transmitter from the postsynaptic cell could indicate to the presynaptic cell that the signal has been received. Because NO diffuses across cell membranes, it could mediate a retrograde transmission function as well as explain how neuronal activity could influence neighboring synapses. Models for activity-dependent modulation include long-term potentiation (LTP) and long-term depression (LTD).

nNOS knockout mice have been used to study the roles of these isoforms in hippocampal LTP (O'Dell et al., 1994) and cerebellar LTD (Linden et al., 1995). Interestingly, these processes are normal in the nNOS knockout mice, and LTP is normal in eNOS knockout mice. However, mice deficient in both nNOS and eNOS (eNOS/nNOS double knockout mice) have abnormal LTP in the stratum radiatum but not the stratum oriens. These results provide genetic evidence that NOS is involved in LTP in stratum radiatum and suggest that the neuronal and endothelial forms may compensate for each other in mice with a single mutation. They further suggest that there is also an NOS-independent component of LTP in stratum radiatum and that LTP in stratum oriens is largely NOS independent.

5.2 Regulation of Neurotransmitter Release

NO generation modulates neurotransmitter release in the cerebral cortex and hippocampus. Infusion of NMDA by microdialysis probes into nNOS- and eNOS knockout mice stimulates production of NO by the remaining isoforms. No differences in basal concentrations or K^+-stimulated glutamate release was found between the various strains. In contrast, NMDA-stimulated glutamate release in the cortex was attenuated in nNOS knockout mice, and NMDA-stimulated GABA release was significantly reduced in all regions of the eNOS knockout mice (Shimizu-Sasamata et al., 1998). These results indicate that the nNOS and eNOS, most likely due to specific neuronal localizations, serve different roles in the modulation of excitatory and inhibitory neurotransmission in the brain. Furthermore, these effects are relevant to excitotoxic amino acid release in core regions and spreading depression-like depolarizations in the periphery of cerebral infarcts.

5.3 Regulation of Blood Flow

Endothelial production of NO throughout the body regulates vascular tone, controlling blood pressure at the systemic level and tissue perfusion at the local level. It is likely that eNOS plays such a role in the brain as well. However, it is also possible that nNOS-derived NO may influence cerebral blood flow. Several adaptations of the cerebrovasculature distinguish it from the rest of the body, as listed in *Table 4-4*.

First, the endothelium of the cerebrovasculature is involved in the blood–brain barrier. Second, it has long been postulated that regional cerebral perfusion is coupled to neuronal activity—that areas of the

Table 4-4
Unique features of cerebrovasculature

Blood–brain barrier
Coupling of blood flow to neuronal metabolism
Autoregulation of cerebral perfusion
Ischemic vasodilation
Generalized changes in cerebral perfusion

brain metabolically active will also derive greater blood flow (Roy and Sherrington, 1890). These variations in blood flow may account for the ability of positron emission tomography scanning to detect metabolically active regions of the brain. Experimentally, stimulation of the whiskers can be used to increase blood flow over the corresponding cortical barrels present in mice, rats, and other animals, showing that coupling is present. Third, the perfusion pressure of the cerebrovasculature is buffered far greater than the systemic blood pressure. Over a wide range of systemic blood pressures, cerebral perfusion remains tightly regulated—a process called autoregulation. Fourth, when the brain is ischemic, blood vessels that supply neighboring regions may vasodilate to maximize perfusion of the affected area. Fifth, there are generalized changes in cerebral perfusion, for example to hypercapnia. NO generated by eNOS and nNOS may play roles in the regulation of regional and generalized cerebral blood flow and compensatory changes during ischemia.

Acetylcholine normally dilates blood vessels by stimulating the production of NO by eNOS. In the aorta of eNOS knockout mice, EDRF activity, defined as endothelium-dependent relaxation to acetylcholine, is absent. However, acetylcholine does dilate pial arterioles in eNOS knockout mice, and this can be blocked by L-NA. Tetrodotoxin blocks this response only in eNOS knockout mice and not wild-type mice. These results suggest that NO made by nNOS may compensate for lack of eNOS (Meng et al., 1996). 1*H*-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one, a soluble guanylyl cyclase inhibitor, attenuated the acetylcholine response of both wild-type and eNOS knockout mice. A selective nNOS inhibitor, 7-NI, blocked the response in eNOS knockout mice only (Meng et al., 1998). This confirms that nNOS compensates for the absence of eNOS in the eNOS knockout mice. This is another example of physiologic compensation where one NOS isoform substitutes for the absence of another.

6 nNOS and Stroke

6.1 Stroke in nNOS Knockout Mice

Malinski et al. (1993) first measured NO production in the brain using a porphyrinic microsensor. While basal levels of NO are in the nanomolar range, the amount rapidly increases to micromolar levels following cerebral ischemia. This increase does not occur in nNOS knockout mice, establishing that nNOS is responsible for the marked rise in NO following ischemia (Huang et al., 1994).

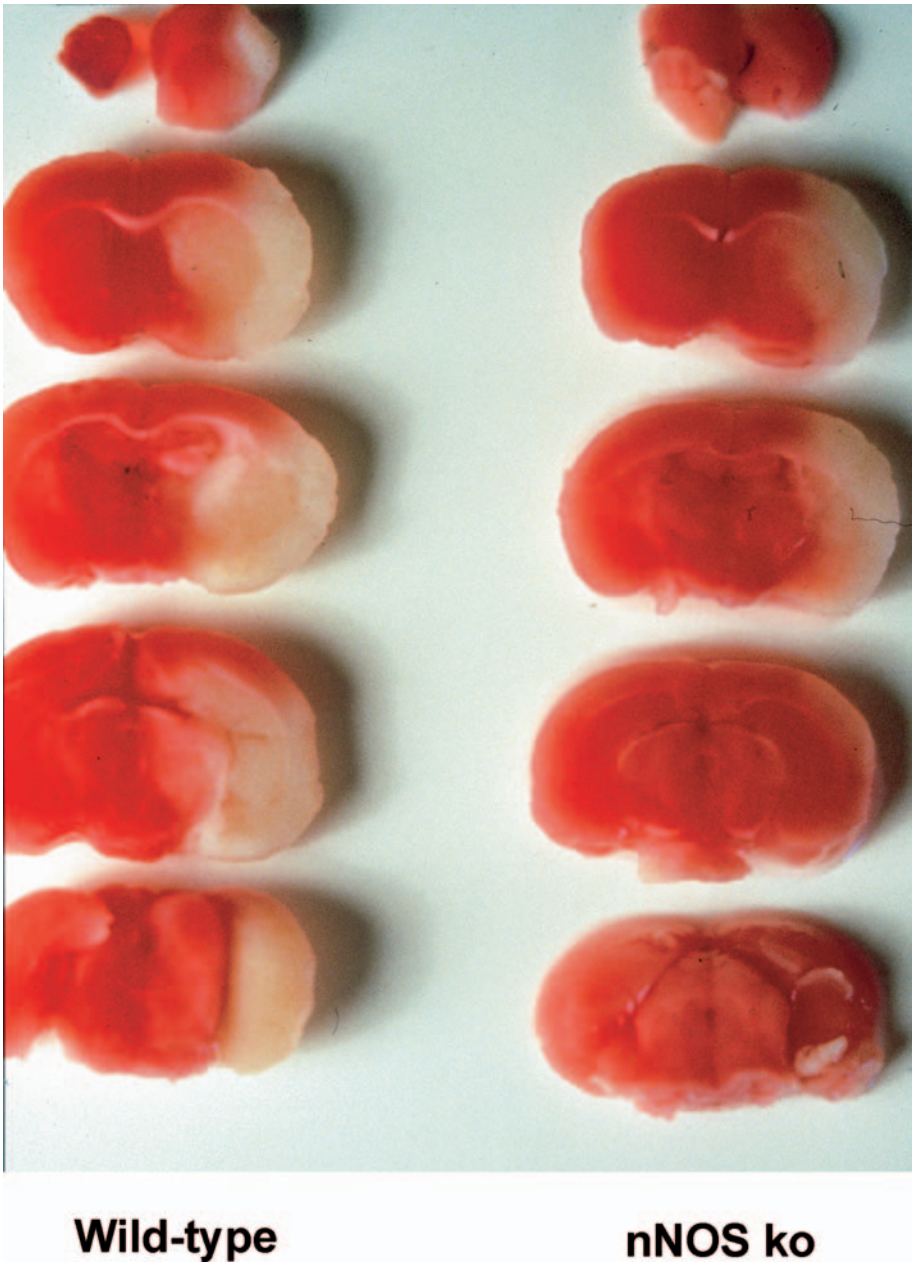
On using the middle cerebral artery occlusion (MCAO) model of focal ischemia, nNOS knockout mice were shown to have a reduction in stroke volume, after transient and permanent ischemia. *Figure 4-2* shows typical infarct sizes seen in coronal sections from brains after MCAO in wild-type and nNOS knockout mice. Living tissue is stained red by 2,3,5-triphenyl tetrazolium chloride (TTC), while the infarct region remains white. Measurement of blood flow by laser Doppler flowmetry shows that the MCAO causes the same reduction in blood flow in both cases, so the nNOS knockout mice do not have smaller strokes because they had better blood flow. They had the same reduction in blood flow, yet they had less damage, implicating nNOS in tissue damage. nNOS also contributes to tissue damage in global ischemia (Panahian et al., 1996). Consistent with these results, selective nNOS inhibitors are also protective.

6.2 Mechanisms of NO Toxicity

6.2.1 Peroxynitrite Formation

NMDA injection into the striatum causes dose- and time-dependent lesions, which are blocked by MK-801 pretreatment. The lesions are smaller in the nNOS knockout mice, consistent with a role for the nNOS isoform in mediating toxicity (Ayata et al., 1997). Nitrotyrosine levels, as detected by immunohistochemistry and electrochemical methods, are increased markedly in the wild-type animals, but not in nNOS

Figure 4-2
Infarct size after MCAO in nNOS knockout mice. Coronal sections are stained with TTC after MCAO. The infarct regions are white. nNOS knockout mice show smaller infarcts after MCAO than wild-type mice



knockout mice, after NMDA injection. Levels of 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid that reflect hydroxyl radical formation are similarly increased in NMDA-lesioned wild-type mice but not nNOS knockout mice. These results establish that peroxynitrite and hydroxyl radicals contribute to tissue damage resulting from nNOS-derived NO.

6.2.2 Activation of PARP

A second mechanism for NO toxicity is activation of PARP, which results in the depletion of cellular energy stores. PARP activation is markedly elevated following focal ischemia in wild-type mice but not in nNOS knockout mice (Endres et al., 1998b). However, NO does not directly activate PARP, but peroxynitrite is a necessary intermediate.

6.2.3 Stimulation of Apoptosis

A third mechanism for NO toxicity is stimulation of apoptosis. NO induces apoptosis in neurons, and this is associated with downregulation of Bcl-2, upregulation of Bax, and activation of caspase-3 (Tamatani et al., 1998). NO also has potential antiapoptotic effects (Nicotera et al., 1999), including inhibition of caspases by S-nitrosylation or by other mechanisms (Melino et al., 1997; Liu and Stamler, 1999; Mannick et al., 1999). Overall, NO plays a key role in apoptotic cell death after ischemia, as evidenced by decreased TUNEL staining and staining for caspase-cleaved actin fragments (fractin) in nNOS knockout mice (Ayata et al., 1997; Elibol et al., 2001).

6.2.4 iNOS and Cerebral Ischemia

The iNOS isoform is not present in the ischemic brain until days later, as glia and inflammatory cells enter the infarct zone. Like nNOS, iNOS contributes to tissue damage after cerebral ischemia. Inhibition of iNOS by selective pharmacologic inhibitors, or gene deletion of iNOS, reduces this late damage (Iadecola et al., 1995, 1997; Zhang et al., 1996).

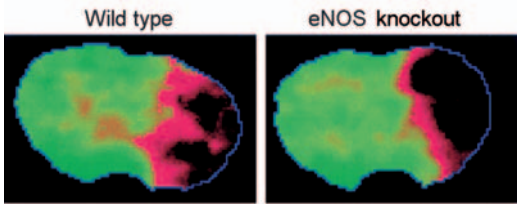
7 eNOS and Stroke

7.1 Stroke in eNOS Knockout Mice

In contrast to the toxic effects of nNOS and iNOS, eNOS mediates vasodilation, inhibition of platelet aggregation, and leukocyte adhesion to endothelium, effects that would all be protective following cerebral ischemia. Indeed, eNOS knockout mice show more injury following MCAO than wild-type mice (Huang et al., 1996). Tissue damage could be reduced by NOS inhibitors, consistent with a cytotoxic role for NO generated by the remaining isoforms (nNOS and iNOS).

In eNOS knockout mice, the enlarged strokes from the MCAO model are associated with a greater effect on blood flow by the filament occlusion of the artery. This has been demonstrated using temporal correlation mapping of blood flow (Lo et al., 1996). Each pixel in a CT scan can be monitored for the kinetics of appearance and disappearance of a contrast agent like iodohexol. Normal blood flow results in a rapid rise in contrast agent, and a prompt washout. Absent flow shows no contrast agent at all. In regions where there is reduced blood flow (the functional penumbra), the contrast agent appears slower than normal, reaches a lower peak, and decays more slowly. Lo coworkers mapped each pixel of a coronal section of the brain following MCAO. Normal blood flow was mapped as green, absent blood flow as black, and present but abnormal blood flow as shades of red (the less blood flow, the darker). As seen in *Figure 4-3*, the regions with normal flow (green) are equivalent, as are the regions at risk (not green). Within this region, eNOS knockout mice showed a greater core ischemic zone, with no blood flow (black) and a smaller ischemic penumbra (red), than wild-type mice. This confirms that the larger strokes in eNOS knockout mice are associated with a more severe effect on blood flow than in wild-type mice.

Figure 4-3
Temporal correlation mapping of blood flow in eNOS knockout mice. Areas of normal blood flow are mapped in green, areas of absent blood flow are mapped in black, and areas of reduced blood flow are mapped in shades of red. Darker regions have less blood flow. Wild-type mice have a definable area of no blood flow (core region) surrounded by areas of reduced blood flow (the ischemic penumbra). eNOS knockout mice show a larger core region and a correspondingly smaller penumbra



7.2 Effects of Statins on eNOS

Several large clinical studies show that HMG-CoA reductase inhibitors (statins) protect against stroke, and this effect is independent of cholesterol lowering. In animal studies, statins augment cerebral blood flow, reduce infarct size, and improve neurologic outcome following MCAO (Yamada et al., 2000; Amin-Hanjani et al., 2001). These effects suggest that statins may increase NO generation by eNOS. eNOS levels appear to be unchanged by statins, but NO generation may be increased by other mechanisms involved in regulation of eNOS activity.

To confirm that statins have effects on eNOS activity, eNOS knockout mice and wild-type mice were treated with statins and subjected to MCAO. In contrast to wild-type mice, eNOS knockout mice showed no change in cerebral blood flow, infarct size, or neurologic improvement to statin treatment (Endres et al., 1998a; Laufs et al., 2000). These results demonstrate that enhanced eNOS activity is required for the neuroprotective effects of statins.

7.3 Mechanisms of eNOS Protection

The outcome of ischemia depends not only on neuronal susceptibility but also on interactions between cerebral blood vessels, neurons, circulating cells, extracellular matrix, and surrounding tissue, together termed the neurovascular unit. The cerebrovascular endothelium and endothelium-derived mediators, such as NO, play key roles in these interactions.

7.3.1 Preservation of Blood Flow

NO generated from both nNOS and eNOS appears to be involved in the regulation of regional cerebral blood flow and coupling of blood flow with metabolic demand. At the most basic level, eNOS allows the cerebrovasculature to adapt to ischemic conditions by vasodilating blood vessels that supply the ischemic penumbra, to maintain perfusion of affected areas. These changes are affected by eNOS gene deletion, as shown earlier in *Figure 4-2* (Lo et al., 1996).

7.3.2 Leukocyte-Endothelial Interactions

eNOS-derived NO suppresses leukocyte-endothelial interactions in vitro and in vivo. These interactions contribute to the mobilization of inflammatory cells to vascular regions prone to atherosclerosis and to

areas of ischemia or infarction (Fukumura et al., 1997; Gidday et al., 1998; Lefer et al., 1999; Scalia et al., 1999; Santizo et al., 2002).

7.3.3 Thrombosis

eNOS-derived NO inhibits platelet aggregation and adhesion to the vessel wall. These effects are important to suppress thrombosis from microinjury and may play roles in protection against thrombotic complications of ischemia and infarction (Provost and Merhi, 1997; Loscalzo, 2001).

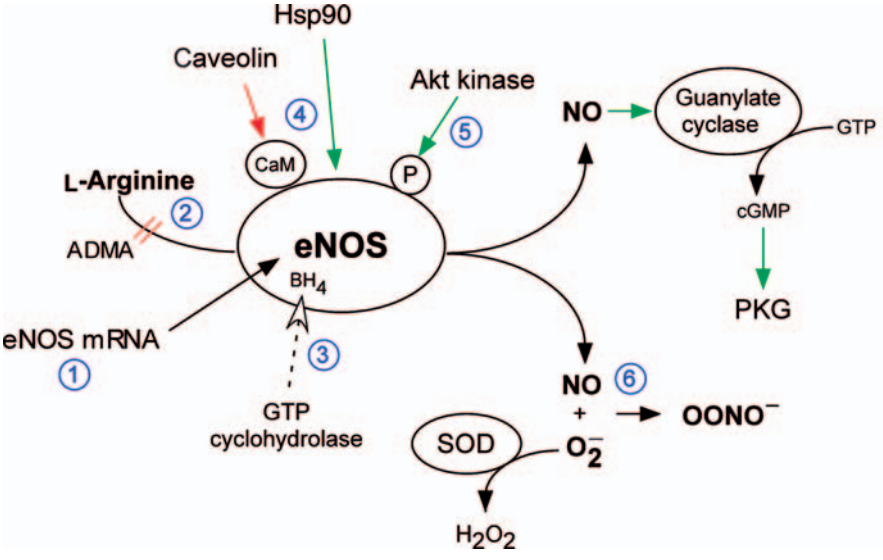
7.4 Endothelial Dysfunction

Endothelial dysfunction, defined as impairment of physiologic endothelium-dependent relaxation, occurs in atherosclerosis, hypertension, hypercholesterolemia, diabetes, and normal aging (Cai and Harrison, 2000; Gimbrone et al., 2000). Impairment of endothelial function occurs before structural changes such as intimal hyperplasia or lipid deposition. Thus, endothelial dysfunction is an upstream event in the pathophysiology of atherosclerosis. The common feature of endothelial dysfunction is a reduction in the amount of bioavailable NO produced in the vessel wall.

There are several overlapping mechanisms for endothelial dysfunction, as shown in *Figure 4-4*. First, changes in eNOS mRNA or protein expression levels can account for changes in eNOS activity (Wang and

Figure 4-4

Molecular mechanisms of endothelial dysfunction. Multiple steps in eNOS generation of bioavailable NO are potential mechanisms for endothelial dysfunction, as described in the text



Marsden, 1995). Second, L-arginine, which is the substrate for NO production, can be limiting in tissues. An endogenous competitive inhibitor, asymmetric dimethylarginine (ADMA) can also reduce endothelial NO production even in the presence of adequate L-arginine levels (Bode-Boger et al., 1996; Cooke, 2000). ADMA levels are increased in obese patients, and they decrease with weight loss (Eid et al., 2004;

Krzyzanowska et al., 2004). Third, eNOS requires FAD, FMN, NADPH, and BH_4 as cofactors. BH_4, whose synthesis is rate-limited by GTP cyclohydrolase, is a particularly important cofactor because in its absence eNOS can generate superoxide anion (Cosentino et al., 1998). Fourth, eNOS requires dimerization and proper intracellular localization to caveolae, mediated in part by interactions with caveolin and hsp90 (Gimbrone, 1989; Shaul, 2002). Fifth, eNOS is phosphorylated at serine 1179 (S1179) by Akt kinase and other kinases (Dimmeler et al., 1999; Fulton et al., 2001). Sixth, NO produced by eNOS may be rapidly inactivated by reaction with superoxide (O_2^-) to form peroxynitrite ($OONO^-$) (Beckman and Koppenol, 1996). This superoxide can be formed by NAD(P)H oxidase (Griendling et al., 2000) or uncoupled eNOS (Cosentino et al., 1998). These mechanisms are not mutually exclusive and have been demonstrated to act in vivo.

8 Next Generation NOS Mice

8.1 Exon 6 nNOS Knockout Mice

The nNOS gene is transcribed from 29 exons into many different splice isoforms. The predominant nNOS isoform is nNOSα. There are nNOS isoforms that lack exon 2 (nNOSβ and nNOSγ), as well as other isoforms that contain in-frame deletions or insertions of other exons. Exon 2 is important to enzymatic function because it encodes the PDZ domain (Brenman et al., 1996, 1997). The original nNOS knockout mice were generated by deleting exon 2 (Huang et al., 1993). Over 95% of the nNOS activity in the brain is absent in these mice. However, the β and γ isoforms of nNOS, normally present at low levels, are still present in the nNOS knockout mice. Because these isoforms lack the PDZ domain, it has been hypothesized that they are soluble isoforms that may play developmental roles.

To address these possibilities, a second nNOS knockout mouse was generated by targeting exon 6, which contains the heme-binding domain required for catalytic activity (Gyurko et al., 2002). The exon 6 nNOS knockout mice, unlike the original nNOS knockout mice, have no functional nNOS catalytic activity. They appear to have a more severe pyloric stenosis phenotype as well as reproductive endocrine abnormalities. These mice should be useful to test which neuronal functions of nNOS-derived NO are due to nNOSα and which ones depend on soluble forms of nNOS present in the exon 2 knockout mice.

8.2 eNOS Mutant Mice

The next generation of NOS mutant mice will likely carry tissue-specific or inducible gene modifications that offer more subtle manipulations than transgenic overexpression or generalized gene knockout. In addition, the specific function of functional domains within the NOS isoforms have been studied by site-specific mutations. For example, phosphorylation of eNOS at S1179 by Akt and other kinases appears to be an important mechanism of regulating eNOS activity. Mutant forms of eNOS, where S1179 is replaced by aspartate (S1179D mutation) and alanine (S1179A mutation), have been generated and characterized in vitro (Dimmeler et al., 1999; Fulton et al., 1999). The S1179D mutant mimics phosphorylation because the aspartate residue is negatively charged like the phosphorylated serine residue; S1179D has greater eNOS activity than wild-type eNOS. In contrast, the S1179A mutation cannot be phosphorylated since the side chain is a methyl group instead of a $-CH_2OH$ group. S1179A has less activity than wild-type eNOS. While these mutant forms of eNOS have been characterized in vitro and in cell culture, their effects in vivo have not been demonstrated. Mice that carry these and similar mutations will likely be useful tools to study the importance of eNOS phosphorylation to vascular function and stroke.

9 Conclusions

NO is a unique biological mediator that plays important roles in the brain. NO is involved in intercellular communication, neurotransmitter release, and synaptic plasticity. It regulates regional cerebral blood flow,

coupling of blood flow to metabolism, and compensatory changes following ischemia. For these neuronal and vascular roles, both nNOS- and eNOS-derived NO appear to be important, as shown by mutant mice that distinguish between the isoforms. After cerebral ischemia, nNOS and iNOS appear to be responsible for the cytotoxic effects of NO, while eNOS is protective. These effects include preservation of blood flow and effects on leukocyte-endothelial interactions and platelet aggregation. In addition, eNOS is important in suppressing atherosclerosis, and its absence is clinically correlated with endothelial dysfunction and increased susceptibility to vessel injury and plaque formation.

An important unanswered question is how NO generated by different NOS isoforms can have different biological effects. That is, how does the cell know that NO is generated by nNOS, iNOS, or eNOS? Part of the answer is in the amount of NO generated. In addition, the precise subcellular localizations of the NOS isoforms (nNOS by its PDZ domain, and eNOS to caveolae) may allow the production of NO in proximity to intended targets. In this sense, NO may not be as freely diffusible as once thought. The biological context of NO generation will also affect its consequences. For example, NO formed in proximity to superoxide will likely form peroxynitrite, while NO formed next to a thiol compound may form a nitrosothiol intermediate that later releases vasoactive NO.

Our understanding of cerebrovascular disease also suggests that atherosclerosis and endothelial dysfunction may affect the function of the cerebrovasculature, and therefore the response to cerebral ischemia. The major pathophysiological mechanisms involved in atherosclerotic lesion formation, including vascular injury, oxidant stress, inflammatory responses, leukocyte-endothelial interactions, and endothelial dysfunction have been demonstrated in larger systemic vessels. The specific gaps in our knowledge are: (1) whether the physiologic and molecular mechanisms of vascular dysfunction in peripheral vessels apply to cerebral vessels and (2) whether the cerebrovasculature shows unique functional or morphologic abnormalities in response to atherosclerosis.

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5 Molecular Mechanisms of DNA Damage and Repair in Ischemic Neuronal Injury

F. Sun · G. Gobbel · W. Li · J. Chen

Abstract: Oxidative DNA damage is a severe consequence of oxidative stress associated with ischemic neuronal injury. The accumulation of DNA lesions, such as oxidative base damage, AP sites, and strand breaks, is an important trigger of both neuronal apoptosis and necrosis. Emerging data suggest that base excision repair (BER), the predominant mechanism for the repair of most types of oxidative DNA damage in neurons, is inducible after oxidative stress and constitutes a novel endogenous mechanism for neuroprotection. This chapter will: (1) review the importance of DNA damage in ischemic neuronal injury; (2) review the signaling pathways leading to cell death triggered by oxidative DNA damage; (3) review the DNA repair mechanisms in the brain; and (4) evaluate the importance of inducible BER as a mechanism for endogenous neuroprotection.

List of Abbreviations: CAD, caspase-activated deoxyribonuclease; AIF, apoptosisinducing factor; CS, Cockayne syndrome; XP, xeroderma Pigmentosum; NER, nucleotide excision repair; PCNA, proliferating cell nuclear antigen; TCR, transcription-coupled repair; APE, AP endonuclease; FEN1, flap endonuclease 1; XRCC1, X-ray cross-complementing group 1; AP site, apurinic/apyrimidinic abasic site; DNA SSB, DNA single-strand break; PANT, DNA polymerase I-mediated biotin-dATP nick translation; ROS, reactive oxygen species; BER, base-excision repair; 8-oxodG, 8-hydroxyl-2´-deoxyguanosine; PARP, poly(ADP-ribose)polymerase; PUMA, p53 upregulated modulator of apoptosis

1 Introduction

Emerging evidence suggests that the accumulation of oxidative DNA damage is an important contributing factor in neuronal cell death induced by cerebral ischemia and reperfusion. Endogenous oxidative damage to nuclear DNA, in the forms of base damage and strand breaks, can be detected in the ischemic brain during stages preceding the manifestations of cell death and is believed to trigger cell death via various intracellular signaling pathways.

An effective DNA repair system is essential for neurons to survive. DNA base excision repair (BER), consisting of short-patch and long-patch repair pathways, is the predominant repair mechanism of oxidative DNA damage in the brain. In neurons, BER is a highly inducible process in response to ischemia or oxidative stress at sublethal levels. Induced BER activity in ischemic brain has been associated with rapid repair of nuclear DNA damage and cell survival. Thus, BER is likely a novel cellular mechanism for endogenous neuroprotection.

This chapter will: (1) review the importance of DNA damage in ischemic neuronal injury; (2) review the signaling pathways leading to cell death triggered by oxidative DNA damage; (3) review the DNA repair mechanisms in the brain; and (4) evaluate the importance of inducible BER as a mechanism for endogenous neuroprotection.

2 Endogenous DNA Damage After Ischemic Brain Injury

2.1 Active Versus Passive DNA Damage

DNA damage resulting from cerebral ischemia and reperfusion involves at least two distinctive mechanisms: an active process mediated by DNA endonuclease(s) and a passive process resulting from direct attacks by reactive oxygen species (ROS) (Charriaut-Marlangue et al., 1995; Chopp et al., 1996). The best-characterized endonuclease-mediated damage is DNA double-strand breaks (DSBs) at nucleosomal junctions, often referred to as apoptotic DNA fragmentation. This type of DNA damage represents a part of the cascade of cellular self-destruction, and it occurs only at a late, irreversible stage of cell injury after ischemia (Linnik et al., 1993; Li et al., 1995; Nitatori et al., 1995; Chen et al., 1998a; MacManus et al., 1999). Cells with internucleosomal DNA fragmentation often exhibit apoptotic morphology, which is synonymous with cell death in vulnerable brain regions after global or focal ischemia (Linnik et al., 1993; MacManus et al., 1993; Li et al., 1995; Charriaut-Marlangue et al., 1996; Chen et al., 1997). Activation of specific endonucleases may

contribute to apoptotic DNA fragmentation. The caspase-activated deoxynuclease (CAD), which is specifically activated by caspase-3 or caspase-7, cleaves DNA between histosomes, resulting in fragments of 180–200 base-pair multiples (Enari et al., 1998). DNA cleaved in this fashion produces a pattern known as "laddering" on DNA gels. Several studies have shown that CAD is activated in vulnerable regions in the brain after transient global or focal ischemia and appears to be responsible for internucleosomal DNA fragmentation in those settings (Cao et al., 2001). However, CAD may not be responsible for all types of apoptotic nuclear changes in neurons after ischemia. MacManus et al. (1999) reported that formation of DNA fragmentation after ischemia is distinct from that found in classical apoptosis, in which most fragmented DNA contains 3′ ends with a recess of eight to ten nucleotides. Furthermore, induction of high-molecular-weight DNA fragmentation, demonstrated by the generation of 50- and 10-kbp fragments, often precedes that of internucleosomal DNA fragmentation in the brain after ischemia (MacManus et al., 1997). These observations suggest that a complex and possibly unique process involving more than one endonuclease may mediate ischemia-induced neuronal DNA fragmentation.

The abundant mitochondrial flavoprotein apoptosis-inducing factor (AIF) is another unique endonuclease that may contribute to ischemia-induced neuronal DNA fragmentation. AIF is released from mitochondria upon receipt of various cell death signals, and it potently promotes apoptosis, mainly through its direct nuclear-degrading activities (Susin et al., 1999; Daugas et al., 2000; Dumont et al., 2000; Ferri and Kroemer, 2000; Vieira et al., 2000). AIF is ubiquitously expressed in the central nervous system and plays a critical role in neuronal apoptosis induced by glutamate toxicity or oxidative stress (Yu et al., 2002; Zhang et al., 2002). As found in studies involving gene transfection or using in vitro cell-free systems, AIF is capable of producing high-molecular-weight DNA fragmentation at the sizes of 50 and 10 kbp in nuclear genomes, and this may directly contribute to its cell-killing effect. In several recent in vivo studies, AIF has been found to redistribute from mitochondria to the nucleus in neurons after transient cerebral ischemia, and this redistribution appears to be correlated with the selective vulnerability of neuronal populations in the brain to ischemic insults (Cao et al., 2003; Zhu et al., 2003; Plesnila et al., 2004).

In contrast to the active DNA degradation mediated by endonucleases, oxidative DNA damage resulting from direct attacks by ROS is a passive process that is independent of any endonuclease activities. Cerebral ischemia-induced oxidative DNA damage consists of highly specific chemical lesions (Liu et al., 1996; Chen et al., 1997; Nagayama et al., 2000). ROS, mainly hydroxyl radicals, attack DNA through direct and indirect mechanisms. For example, hydroxyl radicals can directly extract hydrogen atoms from deoxyribose and ribose ($-CH + {}^{\bullet}OH \rightarrow -C^{\bullet} + H_2O$) or add protons to double bonds ($-C{=}C + 2{}^{\bullet}OH \rightarrow OH-C-C-OH$) (Breen and Murphy, 1995). These processes can result in various DNA lesions, including base modifications such as 8-oxodeoxyguanine (8-oxodG), 8-oxodeoxyadenine (8-oxodA), thymine glycol and thymidine glycol, 5-hydroxy-2′-deoxycytidine (5-OhdC), 5-hydroxy-2′-deoxyuridine (5-OhdU), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FAPYG), 5-hydroxycytosine (5-OHC), and dihydrothymine (DHT). The common DNA lesions formed by DNA oxidation also include apurinic/apyridinic abasic sites (AP sites), single-strand breaks (SSBs), DNA intra- or interstrand crosslinks, DNA–protein crosslinks, and damage to the deoxyribose moiety (Imlay and Linn, 1988; Halliwell and Aruoma, 1991). Alternatively, DNA damage can be induced indirectly by secondary reactive species formed from the reaction of free radicals with lipids or proteins. Peroxidation of polyunsaturated lipids generates substances that possess DNA-damaging potential. These include lipid hydroperoxides and various species with unpaired electrons such as the alkoxyl and peroxyl radicals (Burcham, 1998).

The induction pattern of oxidative DNA damage after cerebral ischemia has several distinctive characteristics compared to endonuclease-mediated DNA damage. An important feature of ischemia-induced oxidative DNA damage is its early onset. While endonuclease-mediated DNA damage is not detectable within the first several hours after ischemia, oxidative DNA damage may occur as early as minutes after transient cerebral ischemia. Several types of oxidative DNA lesions, such as 8-oxodG, AP sites, and SSBs, have been detected in the brain either at the beginning of postischemic reperfusion or minutes into the reperfusion phase (Liu et al., 1996; Chen et al., 1997; Cui et al., 1999; Huang et al., 2000). One of the first pieces of supportive evidence comes from a study by Liu et al. (1996). Mice were subject to 30 min of forebrain ischemia followed by up to 24 h of reperfusion, and cortical brain samples were collected at the end of ischemia and after 8–24 h of reperfusion. An increase in oxidative damage to DNA bases was detected

starting approximately 10 min after reperfusion. The presence of oxidative changes is consistent with the damage being caused by free radicals, and the fact that the time of greatest increase occurred during reperfusion suggests that most of the free radicals were generated during reoxygenation. In this same study, an increase in DNA mutations was also detected using an interesting approach involving the "Big Blue" transgenic mouse. Although no significant change in mutation frequency occurred immediately after ischemia, a greater than fivefold increase occurred at 8 h of reperfusion and a threefold increase was seen at 24 h after insult. A similar increase in DNA base damage was reported in the rat after 30 min of focal ischemia, and the damage was observed within the first half-hour of reperfusion, whereas TUNEL-labeling was not detected until 2 days after insults (Cui et al., 2000). Further evidence that ischemia and reperfusion can cause DNA damage comes from a study of SSBs and DSBs in rat brain following middle cerebral artery (MCA) occlusion and reperfusion (Chen et al., 1997). As in the study of base lesions and mutagenesis, significant increases in SSBs did not become apparent until 1 min into reperfusion, suggesting that reoxygenation and possibly free radicals were likely contributors to the formation of strand breaks.

The spatial distribution of these oxidative lesions during early reperfusion periods does not predict regional selective vulnerability to ischemic cell death. This was studied using the rat focal ischemia model, where 1 h of MCA occlusion results in infarction in the caudate-putamen but not in the frontal-parietal cortex. The formation of 8-oxodG, AP sites, and SSBs was found to be markedly increased in both regions during the first 2–4 h of reperfusion (Lan et al., 2003). Unlike in transient ischemia, induction of oxidative DNA lesions after permanent focal ischemia is limited to the peri-infarct regions where oxygen tension is partially preserved (Nagayama et al., 2000), again demonstrating the importance of the generation of ROS in this damaging process.

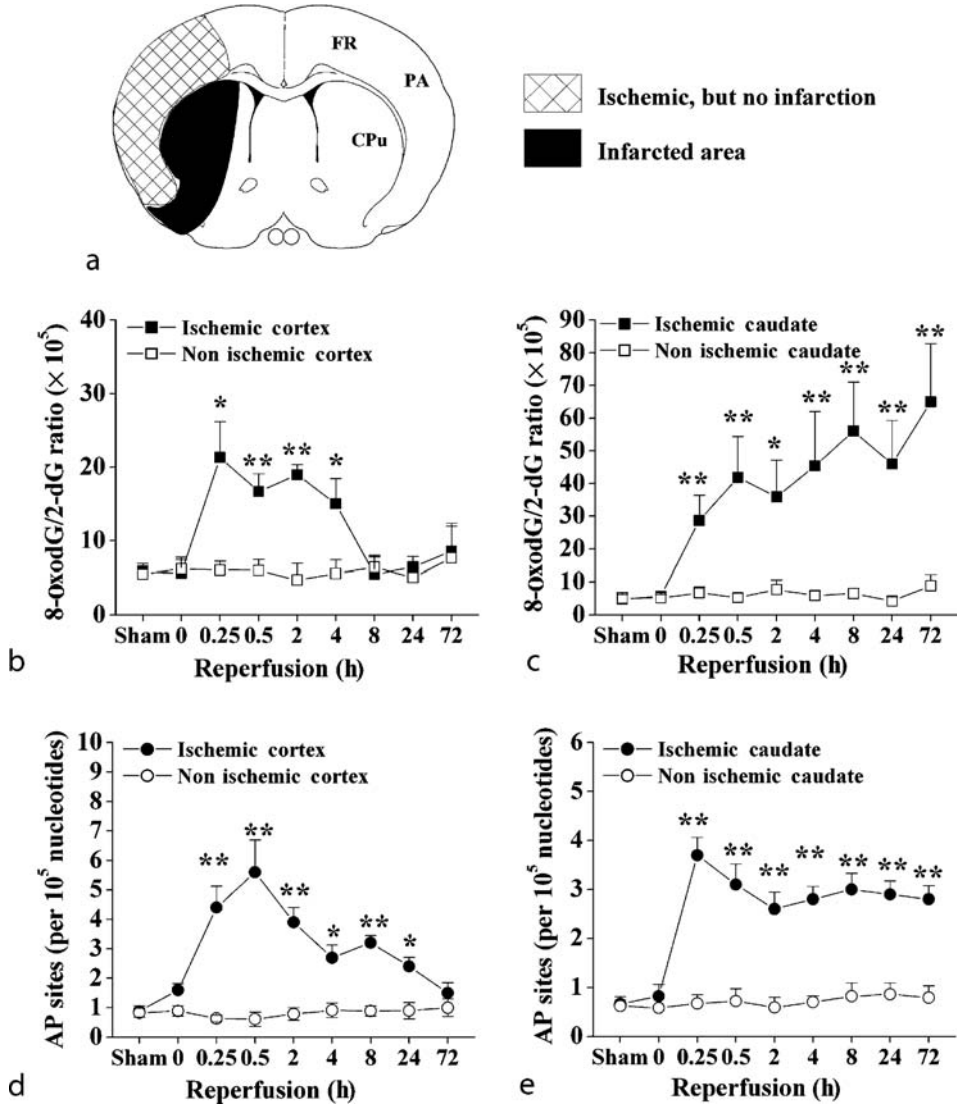
In both transient and permanent ischemia settings, the induction of oxidative DNA damage is at least partially reversible, and this reversibility closely correlates with the outcome of the injured tissues after ischemia (*Figure 5-1*). As an important feature, oxidative DNA damage is found to be repairable in cells that eventually survive ischemia (Chen et al., 1997; Cui et al., 1999; Lan et al., 2003). In contrast, oxidative lesions are accumulated in cells of brain regions that ultimately develop DNA fragmentation and cell death (Chen et al., 1997; Cui et al., 2000). Thus, accumulation of oxidative DNA damage is likely an important contributing factor in ischemic neuronal death (Eliasson et al., 1997; Endres et al., 1997; Fujimura et al., 1999; Cui et al., 2000; Cao et al., 2001).

2.2 Oxidative DNA Damage as a Trigger of Neuronal Cell Death

Damage to DNA is one of the most crucial events in the cytotoxicity of ROS (Schraufstatter et al., 1988; Halliwell and Aruoma, 1991). Several types of oxidative DNA damage may have specific detrimental effects on the fate of ischemic neurons. 8-oxodG, the most prevalent oxidative base modification resulting from direct attacks by hydroxyl radicals, has been associated with gene mutagenesis, giving rise predominantly to GC → TA transversions (Gajewski et al., 1990; Dizdaroglu, 1991; Grollman and Moriya, 1993). Genes that are enriched in the formation of 8-oxodG may partially or completely lose their functional properties (Schneider et al., 1990; Mazzarello et al., 1992; Cui et al., 1999), although the immediate impact of the mutagenesis due to 8-oxodG on cell survival is unclear. In contrast, several types of DNA lesions are found to be potent inducers of cell death (Janssen et al., 1993; Demple and Harrison, 1994). For instance, thymine glycol, thymidine glycol, and FAPYG are known cytotoxic lesions (Imlay and Linn, 1988). AP sites and SSBs, among the most prevalent oxidative lesions formed in neurons after ischemia, represent the type of DNA lesions that have potent cell-killing effects. AP sites are generated by spontaneous base loss, base loss induced by hydroxyl radicals, or as intermediates of BER of base lesions by specific *N*-glycosylases (Demple and Harrison, 1994). AP sites, upon accumulation, prevent the process of DNA synthesis or gene transcription through the lesion sites and thus are directly lethal to cells (Schaaper and Loeb, 1981; Moran and Wallace, 1985; Janssen et al., 1993). DNA strand damage, such as SSBs, is also a potent blocker of DNA synthesis and gene transcription in cells. The accumulation of DNA strand breaks may directly trigger cell death through several intracellular prodeath signaling pathways (Nelson and Kastan, 1994; Miyashita and Reed, 1995; Payne et al., 1995; Eliasson et al., 1997; Endres et al., 1997). Several studies have shown that AP

Figure 5-1

The reversibility of oxidative DNA damage correlates with the outcome of ischemic brain injury. (a) Schematic diagram depicts the approximate distribution of infarction in the rat brain 72 h after 1 h of MCAO. The cortical and caudate regions were sampled separately for the measurement of DNA damage at various time points after ischemia. Frontal cortex (FR), parietal cortex (PA), caudate putamen (Cpu). Temporal profiles of induction of 8-oxodG in nuclear DNA of the cortex (b) and caudate (c) after 1 h of MCAO, measured using high-performance liquid chromatography with electrochemical detection. Temporal profiles of induction of AP site in nuclear DNA of the cortex (d) and caudate (e) after 1 h of MCAO, measured using the aldehyde reactive probe (ARP) assay. Data are mean ± SEM ($n = 6$–8 per time point). *$p < 0.05$; **$p < 0.01$ versus contralateral nonischemic hemispheres



sites and SSBs are accumulated in ischemic neurons that eventually develop DNA fragmentation and apoptotic cell death (Chen et al., 1997; Cui et al., 2000; Huang et al., 2000; Lan et al., 2003), suggesting that oxidative DNA damage may be an important trigger of ischemic neuronal cell death.

Emerging evidence suggests not only that DNA damage occurs as a relatively early event following cerebral hypoxia and ischemia but also that such damage actually contributes to neuronal cell injury and death. Evidence supporting a role for DNA modifications in neuronal injury comes from studies of the effect of DNA damaging agents on neuronal viability. For example, DNA appears to be the primary target of ionizing radiation and cause of subsequent cell death, and when cortical neurons growing in vitro are treated with doses of 2–16 Gy ionizing radiation, they undergo apoptosis (Gobbel et al., 1998). Other DNA damaging agents that have been shown to cause neuronal apoptosis include ultraviolet (UV) irradiation, cytosine arabinoside, and camptothecin (Morris and Geller, 1996; Park et al., 1998a and 1998b). Interestingly, cell death occurs by nonapoptotic mechanisms like necrosis after more severe DNA damage due to higher doses of ionizing radiation (Gobbel and Chan, 2001). This finding has significant implications because it has been reported that severe ischemic insults are dominated by a necrotic response whereas less severe insults produce apoptosis (Du et al., 1996). Thus, DNA damage could be a contributing factor in both neuronal apoptosis and necrosis.

The importance of DNA integrity to neuronal viability is also shown in the pathologic changes experienced by individuals missing critical DNA repair proteins. Cockayne syndrome (CS) and xeroderma pigmentosum (XP) are two genetic disorders that are the result of deficits in DNA repair (Rapin et al., 2000). Patients with either CS or XP are extremely sensitive to sun exposure as a result of faulty DNA repair. XP has seven variant forms referred to as complementation groups, and the precise phenotype is dependent on the exact underlying genetic mutation. CS and XP patients in complementation groups C (XPC) and D (XPD) typically have extensive neurological deficits including mental retardation, ataxia, and progressive cognitive impairment (Rapin et al., 2000). In the case of CS, postnatal brain growth is severely inhibited, and there is neuronal loss and apoptosis in the cortex, cerebellum, and basal ganglia. The neurological disorder of XPC/D patients is accompanied by neuronal loss, cortical atrophy, and microcephaly. An apparent difference between XP and CS is that while XP results from degeneration of developed neurons, some of the neurological deficits of CS may arise from myelinopathy (Rapin et al., 2000). The disorder associated with XPC/D results from deficits in nucleotide excision repair (NER) of DNA. In XPC, this is due to inability to detect DNA lesions; in XPD, it is due to the lack of helicase involved in excision of lesions (Cleaver et al., 1999; Norgauer et al., 2003). CS results from deficits in proteins that couple the transcription process to repair of damaged genes (Cleaver et al., 1999). Another genetic disorder that involves defects in DNA repair is ataxia-telangiectasia (AT). Patients with AT are defective in a protein known as ataxia-telangiectasia mutated (ATM), which is responsible for the recognition of and response to DNA DSBs (Gately et al., 1998). Cerebellar neurodegeneration is one of the most apparent abnormalities in autopsies of these patients, but neuronal atrophy occurs throughout the brain (Broder, 1985).

Thus, three separate lines of evidence support the hypothesis that DNA damage resulting from ischemia can cause decreases in neuronal viability. First, ischemia and reperfusion are accompanied by DNA alterations and increased expression of gene products important in the cellular response to DNA damage. Second, agents that preferentially induce DNA damage can lead to neuronal apoptosis and necrosis. Third, the loss of DNA repair proteins appears to make the brain more vulnerable to neuronal loss.

2.3 Prodeath Signaling Pathways Triggered by DNA Damage

A number of studies have suggested that blockage of DNA damage-triggered prodeath signaling pathways may offer neuroprotection against ischemic brain injury (Eliasson et al., 1997; Endres et al., 1997; Lo et al., 1998; Culmsee et al., 2001; O'Hare et al., 2002; Wang et al., 2002; Iwashita et al., 2004). In neurons, the mode of cell death induced by oxidative DNA damage appears to be largely dependent on the severity of the insult. While low doses of a DNA-damaging agent result in apoptosis, oxidative insults at high doses lead mainly to necrosis, which is characterized by energy failure and the loss of cell membrane integrity in the early stages of neuronal death (Gobbel et al., 1998).

A well-characterized signaling mechanism that mediates DNA strand break-induced neuronal death is the overactivation of poly(ADP-ribose) polymerase-1 (PARP-1) and subsequent depletion of cellular NAD^+. DNA strand breaks, especially SSBs, are potent PARP activators in neurons. With excessive DNA damage, continuous activation of PARP results in dramatic decreases in cellular NAD^+ levels and

drastic alterations in glycolysis (Zhang et al., 1994). This sequentially causes a decrease in ATP, a depletion of reduced glutathione, an increase in cytosolic Ca^{2+}, and eventually cell death (Payne et al., 1995). Recently, the cross talk between PARP-1 activation and mitochondrial release of apoptosis-inducing factor has been proposed as an important mechanism underlying PARP-1-dependent neuronal apoptosis (Yu et al., 2002).

In fact, neuronal apoptosis induced by genomic DNA damage may involve very complex mechanisms. Much of our current knowledge about DNA damage-responsive signaling is gained from studies employing irradiation, UV, and DNA-damaging chemicals as signaling inducers. Although our understanding is limited, such information nevertheless provides a guideline for helping us understand how DNA damage may trigger the cell death pathways in neurons.

Signaling following irradiation-induced DNA damage, such as DSBs and AP sites, is mediated by members of the phosphatidylinositol (PI) 3-kinase related kinases (PIKKs), specifically ATM, ATM and Rad3 related (ATR), and DNA-dependent protein kinase catalytic subunits (DNA-PK_{CS}) (Abraham, 2001). Other members of the PIKK family include mammalian target of rapamycin (mTOR), suppressor of morphogenesis in genitalia-1 (SMG-1), and transformation/transcription domain-associated protein (TRRAP) (Abraham, 2004). ATM has kinase activity that can be activated directly by DNA damage and is required for radiation-induced apoptosis of certain neuronal populations within the developing nervous system (Gately et al., 1998; Herzog et al., 1998; Chong et al., 2000). One well-known downstream target of ATM activation is p53, which becomes phosphorylated at serine-15, leading to stabilization and upregulation (Bean and Stark, 2001). ATM also acts as a kinase on several other downstream targets involved in the response to DNA damage. These targets include c-Abl, breast cancer susceptibility gene (BRCA1), the checkpoint kinases Chk1 and Chk2, the histone protein H2A variant known as H2AX, 53BP1 (p53 binding protein-1), and p95/nbs1 (Baskaran et al., 1997; Shafman et al., 1997; Chen et al., 1999; Lim et al., 2000; Foray et al., 2002; Tauchi et al., 2002; Gatei et al., 2003; Motoyama and Naka, 2004). The c-Abl protein is a tyrosine kinase that can activate p73; p73 is a homolog of p53 and can transactivate other downstream apoptotic effectors leading to p53-independent apoptosis (Yuan et al., 1999). The importance of c-Abl in DNA damage signaling is shown by experiments demonstrating that apoptosis following treatment with ionizing radiation is blocked in cells lacking c-Abl (Kharbanda et al., 1998; Rich et al., 2000). BRCA-1 is a required cofactor for phosphorylation of several targets by ATM and ATR, including p53, Chk2, and p95/nbs1 (Foray et al., 2003). Chk1 and Chk2 are important for activation of cell cycle checkpoints (Yang et al., 2003). Following phosphorylation by ATM or DNA-PK, H2AX recruits repair proteins to the sites of DNA damage, including BRCA-1, p95/nbs1, and 53BP1 (Yang et al., 2003). The p95/nbs1 protein, which is the product of the *NBS1* gene, is mutated in Nijmegen break syndrome and plays a crucial role in homologous recombination repair. ATM is cleaved by caspase-3 as part of the apoptotic process, and as a result its signaling abilities are lost while its DNA binding capabilities remain intact so that it acts to inhibit DNA repair (Smith et al., 1999).

Like ATM, ATR is important in the initiation of apoptotic signaling. ATR appears to be activated by damage generally produced by ultraviolet radiation, namely pyrimidine dimerization and adduct formation (Sinha and Hader, 2002; Yang et al., 2003). The targets of ATR are similar to those of ATM, and the variation in downstream signaling by these two molecules appears to be brought about by differences in their location within the nucleus and differences in their specific activity toward the various target molecules (Abraham, 2001). DNA-PK_{CS} is one molecule in the complex known as DNA-PK, which also includes Ku70/80. The Ku proteins appear to be the ones that actually bind to the free ends of DSBs and then recruit DNA-PK_{CS} to form the active complex (Smith and Jackson, 1999; Yang et al., 2003). The association of DNA-PK_{CS} with Ku proteins allows for autophosphorylation, which is required for the form of DNA repair commonly referred to as nonhomologous end-joining (NHEJ) (Chan et al., 2002; Douglas et al., 2002).

The three PIKKs discussed earlier, ATM, ATR, and DNA-PK_{CS}, are all considered to be primarily important in the sensing and repair of DSBs. However, DSBs have not yet been detected as an early event in response to cerebral ischemia. Nevertheless, experimental evidence suggests that these molecules are activated during ischemia and may contribute to neuronal injury. Hypoxia and reoxygenation lead to activation of ATR and ATM, respectively, and to phosphorylation of downstream targets (Hammond et al., 2002, 2003, 2004). In addition, nitric oxide (NO) generated during inflammation can lead to ATM/ATR-dependent phosphorylation of p53 (Hofseth et al., 2003). Whether NO generated during cerebral ischemia

has a similar effect on p53 is unknown, but NO is reported to enhance apoptosis following ionizing radiation and DNA damage (Wang et al., 2003). Although there is, as yet, no direct evidence that DNA-PK_{CS} plays a role in ischemia, it has been reported that ischemic preconditioning leads to an increase in expression of Ku70, a DNA-PK_{CS}-binding protein, in CA1 neurons of the hippocampus, and that this expression is associated with resistance to ischemia-induced cell death (Sugawara et al., 2001).

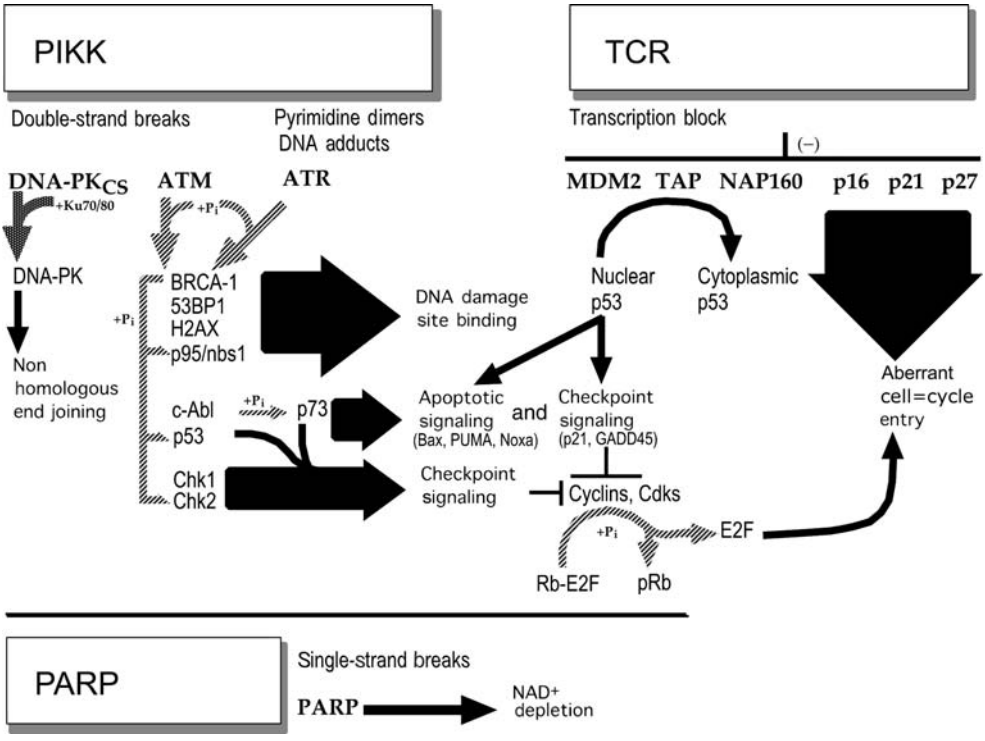
A number of the molecules targeted for phosphorylation by the PIKKs, including BRCA1, Chk1, Chk2, and p53, contribute to checkpoint signaling during cell division. Initially, it appears that such molecules are not likely to play a significant role in the response of postmitotic neurons to DNA damage. However, it has been suggested that apoptosis may, in some cases, be due to aberrant reentry into cell division (Liu and Green, 2001). The finding that ischemia can lead to expression and/or activation of cell cycle proteins such as cyclin D, CDK3, CDK5, p21, phospho-Rb, E2F, and proliferating cell nuclear antigen (PCNA) certainly lends credence to this hypothesis (Guegan et al., 1997; Tomasevic et al., 1998; Hayashi et al., 2000; Osuga et al., 2000; Katchanov et al., 2001; Becker and Bonni, 2004). Pharmacologic inhibitors of CDK as well as dominant negative forms of CDK4/6 protect neurons against DNA-damaging agents like camptothecin (Park et al., 1998a). Also, expression of an endogenous CDK inhibitor, $p16^{INK4a}$, can also protect against DNA damage-induced neuronal apoptosis (Park et al., 1998a). These findings, along with the report that ischemia can decrease the expression of $p16^{INK4a}$, have led to a proposed pathway of aberrant activation of cell cycle proteins and apoptosis. Loss of expression of CDK inhibitors such as p16, p27, or p21 could lead to activation of complexes cdk4/5-cyclin D and cdk2-cyclin E (O'Hare et al., 2002). These activated complexes can then phosphorylate pRb, the retinoblastoma protein, which then allows E2F to become active. In support of this idea, animal studies suggest that hippocampal neurons expressing increased levels of p21 are protected from ischemic injury (Tomasevic et al., 1998). Activation of Chk1 and Chk2 would be expected to block cell cycle progression both by phosphorylation of p53 and degradation of Cdc25A, leading to activation of the G1/S and G2/M checkpoints (Ahn et al., 2000; Chehab et al., 2000; Hirao et al., 2000; Matsuoka et al., 2000; Xiao et al., 2003). Chk2 can also phosphorylate E2F, leading to apoptosis (Steven et al., 2003).

The p53 protein is perhaps the most prominently noted molecule in studies of DNA damage and is sometimes regarded as the "universal sensor of genotoxic stress" (Yang et al., 1998; Yang et al., 2003). However, the role of p53 in response to ischemic injury is still controversial. Increased p53 expression is seen in animal models after focal ischemia (Li et al., 1994). Furthermore, chemical inhibition of p53 expression can protect neurons growing in vitro from the effects of DNA-damaging agents and can reduce the severity of brain injury following temporary focal ischemia in mice (Culmsee et al., 2001). However, whereas Crumrine et al. (1994) reported that brain injury following permanent focal ischemia was ameliorated in p53-deficient mice, another group reported that transient focal ischemia produced a greater infarct size in these same mice (Maeda et al., 2001). This is not entirely surprising since p53 can block cell cycle progression by transactivation of p21 (which would presumably block aberrant cell cycle production and apoptosis) and also can induce upregulation of proapoptotic proteins including Bax, PUMA, and Noxa (Motoyama and Naka, 2004). This upregulation can, in turn, lead to activation of other downstream apoptotic effectors such as caspase-3 (Cregan et al., 1999; Xiang et al., 1998; Jordan et al., 1997; Inamura et al., 2000).

Another mechanism besides the PIKKs that the cell uses to sense and respond to DNA damage is commonly referred to as transcription-coupled repair (TCR). RNA polymerase II (Pol II), because of its role in transcription of genomic DNA into mRNA, is a strong candidate for sensing DNA damage because of its constant surveillance of the genome. When Pol II encounters DNA damage in the form of interstrand crosslinks, bulky adducts, and AP site, the DNA can be distorted in such a way as to block further transcription. Such lesions need to be repaired, or they may result in cell death, either due to loss of proteins critical to survival or via p53-mediated apoptosis signaling (Ljungman and Lane, 2004). The mechanism by which p53 is elevated followed blockage of transcription is not clear. One possibility is that it is related to decreases in levels of MDM2 that occur following transcription blockade (Ljungman and Lane, 2004). MDM2 is a key component required for the nuclear export of p53 (Friedman and Levine, 1998), so decreases in MDM2 would lead to nuclear accumulation. Another possibility is that p53 accumulation is related to altered export of mRNA from the nucleus. Inhibition of TAP and NUP160, critical components in

Figure 5-2

Established and hypothetical mechanisms of molecular signaling following DNA damage in neurons. The primary mechanism that has been identified for detection and downstream signaling of DNA damage in cells involves the phosphatidylinositol (PI) 3-kinase related kinases (PIKKs), specifically DNA-PK_{CS} and ATM. SSBs can be detected by direct binding of PARP, resulting in extensive ADP-ribosylation of protein targets and NAD^+ depletion. DNA adducts and pyrimidine dimers can be detected by the ATR PIKK. This as well as other forms of damage, such as AP site, that results in significant alterations in DNA structure can also inhibit RNA transcription and induce TCR. Inhibition of transcription may cause downregulation of critical molecules, such as those involved in p53 nuclear export and cell cycle inhibition, potentially leading to apoptotic p53 signaling and/or aberrant entry into the cell cycle. There is significant downstream crosstalk between all these signaling pathways, and whether the outcome is repair or cell death likely depends on both the extent and duration of the damage



nuclear export of mRNA, can lead to blockade of export of proteins containing nuclear-export signals, such as p53 (O'Hagan and Ljungman, 2004) (*Figure 5-2*).

3 Repair Mechanisms of Oxidative DNA Damage

Several DNA repair mechanisms have been described in mammalian systems. They can generally be divided into three categories: (1) direct reversal of DNA damage, in which a single enzymatic step is able to restore the normal state of DNA. This form of repair is responsible for the repair of O6-alkylguanine, UV-induced pyrimidine dimmers, ligation of DNA SSBs and purine insertion (Mazzarello et al., 1992); (2) DNA excision repair, which includes BER and NER. Both pathways require the participation of a number of repair enzymes; and (3) daughter-strand gap repair or postreplicative repair. This occurs in dividing cells when

bulky lesions are left in the DNA template. Replication of one strand is stopped in front of the lesion and reinitiated in the undamaged region beyond the lesion, while the gap upstream from the damage is repaired by the recombinant process (Kaufmann, 1989).

3.1 Mechanism of the Base Excision Repair Pathway

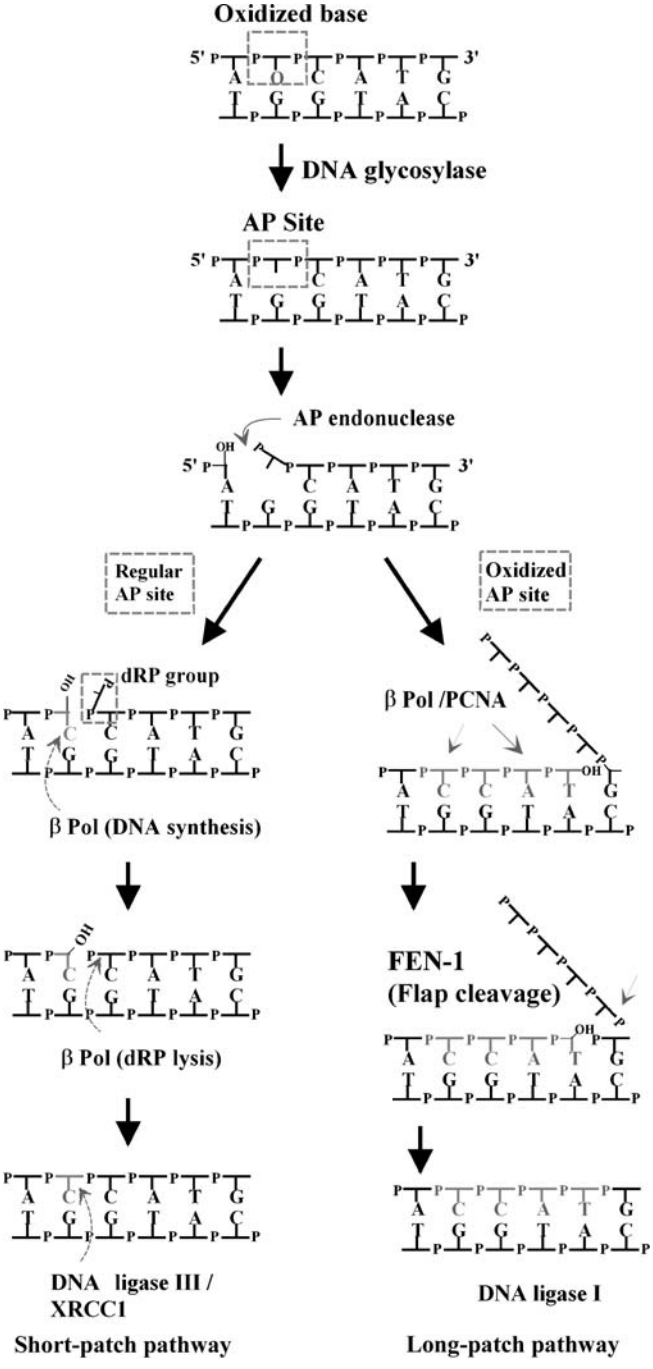
Most, if not all, of the DNA lesions induced by ROS are repaired via the BER pathway (*Figure 5-3*) in mammalian cells (Demple and Harrison, 1994; Frosina et al., 1996; Lindahl et al., 1997). The recent rapid advances in studies of both prokaryotes and eukaryotes have explored the biochemical mechanisms of this important repair pathway in great detail. In BER, repair is initiated by various DNA glycosylases, which excise altered bases by hydrolytic cleavage of the base–sugar bond, generating a regular apurinic/apyrimidinic site in double-stranded DNA. The deoxyribose residue at the regular AP site is susceptible to cleavage by β-elimination. Thus, for the repair of regular AP sites, the process involves the sequential single-nucleotide gap-filling steps. This process is also referred to as the "short-patch" BER pathway, in which the 5′-acting AP endonuclease (APE) cleaves the phosphodiester backbone 5′ to the AP site, generating a 3′-hydroxyl group and a 5′ terminal deoxyribose-phosphate (dRP) moiety. DNA polymerase β (β-pol) then synthesizes a single nucleotide to fill the gap, and this is followed by excision of the dRP moiety by β-pol via β-elimination. Finally, a DNA ligase (DNA ligase III/XRCC1) seals the resulting nick between the newly synthesized nucleotide and the DNA template. The DNA ligase does not act until the 5′-dRP group is removed (Srivastava et al., 1998). Indeed, the rate-limiting step in the short-patch repair pathway is the removal of the dRP group, catalyzed by β-pol. This conclusion is based on the measurement of kinetic constants of purified BER enzymes, in which the velocity of dRP lyase (β-pol) was found to be 6- and 5.3-fold lower than that of DNA polymerase (β-pol) and DNA ligase I, respectively, but very similar to that of the overall BER activity (Srivastava et al., 1998).

The repair of oxidative lesions in mammalian cells via the BER pathway is complicated by the fact that certain AP sites altered by ROS cannot be repaired by β-pol in the same way as regular AP sites can. These include sites that are oxidized (by proton extraction from the sugar ring) and reduced (by addition of proton to the deoxyribose or ribose). These altered sites can be incised by APE, but subsequent excision of the altered sugar–phosphate residue is refractory to β-elimination (Demple and Harrison, 1994). However, these lesions are efficiently repaired via the alternative BER pathway, the so-called "long-patch" BER pathway, which involves the synthesis of two to six, but most frequently four, nucleotides (Frosina et al., 1996; Klungland and Lindahl, 1997). In this repair process, following the incision by APE at the phosphodiester backbone, a short 5′-dRP-containing oligomer downstream of the AP site (typically, 5′-dRP-trinucleotide), but not the dRP group alone, is formed as the major excision product (Dianov et al., 1999). The nucleotide gap at the lesion site is filled by strand displacement DNA synthesis via the action of β-pol (Dianov et al., 1999; Horton et al., 2000), and the nick is subsequently sealed by DNA ligase I (Klungland and Lindahl, 1997). Several enzymes appear to be essential for the long-patch pathway. The structure-specific flap endonuclease 1 (FEN1) and β-pol are essential enzymes and cooperate in the excision and DNA-synthesis steps (Kim et al., 1998). FEN1, which preferentially cleaves unannealed 5′-flap structures in DNA, is responsible for the excision of the dRP-oligomer (Dianov et al., 1999), and it also stimulates strand displacement DNA synthesis by β-pol (Prasad et al., 2000). β-pol-mediated strand displacement DNA synthesis is required for FEN1 to specify the sizes of the excision product (Prasad et al., 2000). Two other proteins, PCNA and replication protein A (RPA), which are involved in the NER pathway, are also important, although not essential, components of the protein complex responsible for long-patch BER (Frosina et al., 1996; Klungland and Lindahl, 1997; DeMott et al., 1998; Tom et al., 2000). PCNA strongly stimulates the 5′-flap cleavage activity of FEN1 (Wu et al., 1996; Gary et al., 1999). This effect by PCNA appears to be achieved by stabilizing the binding of FEN1 to the site of cleavage on its polymer substrate. RPA strongly stimulates the final steps in long-patch repair, probably by interacting with FEN1 and DNA ligase 1, although the precise mechanism is unclear.

Finally, it has been suggested that PARP and X-ray cross-complementing group 1 (XRCC1) also have a regulatory role in both short-patch and long-patch BER (Thompson and West, 2000). XRCC1 mediates

Figure 5-3

BER pathways. The schematic diagram illustrates the biochemical steps involved in short-patch and long-patach BER pathways. Apurinic/apyrimidinic abasic site (AP site), deoxyribose-phosphate moiety (dRP), DNA polymerase-beta (β-pol), structure-specific flap endonuclease 1 (FEN1), proliferating cell nuclear antigen (PCNA); X-ray cross-complementing group 1 (XRCC1)



BER by forming repair complexes with β-pol, PARP, and DNA ligase III (Caldecott et al., 1996; Kubota et al., 1996; Masson et al., 1998). XRCC1 stimulates the activity of DNA ligase III in the ligation step of BER (Cappelli et al., 1997). Since both short-patch and long-patch BER generate SSBs in DNA as repair intermediates, and PARP possesses a specific domain for binding to SSBs, it has been proposed that PARP provides a nick-sensing function and subsequently recruits the BER complex to the nick through interacting with XRCC1 (Caldecott et al., 1996; Wilson and Thompson, 1997; Trucco et al., 1999).

The precise mechanism by which the cell chooses between short-patch and long-patch BER pathways for repairing oxidative lesions is not fully understood. Prasad et al. (2000) suggest that the 5′-dRP-containing intermediate generated by APE incision may be involved in the choice of BER subpathway. Accordingly, if the 5′-dRP residue is not removed and DNA ligation is not completed, continued DNA synthesis could lead to strand displacement and FEN1 excision, resulting in the long-patch BER. In the same scenario, if the sugar in the AP site is altered, as in oxidized AP sites, and it is not a substrate for dRP lyase, long-patch BER would be the preferred repair mechanism (Klungland and Lindahl, 1997). The long-patch pathway is particularly significant in the repair of DNA damage induced by oxidative stress (Horton et al., 2000), since the oxidized AP site appears to be the prominent type of AP site accumulated in the ischemic brain (Nagayama et al., 2000).

3.2 Regulation of Neuronal Base Excision Repair Activity with Aging

A causative relationship between the accumulation of endogenous oxidative DNA damage and deficiency in neuronal BER activity is well illustrated in aging studies. A number of studies with human or rodent subjects have found markedly age-dependent increases in the contents of oxidative DNA damage in the brain and other organ tissues (Mecocci et al., 1993; Hirano et al., 1996; Kaneko et al., 1996; Hudson et al., 1998; Izzotti et al., 1999; Barja and Herrero, 2000; Hamilton et al., 2001). Compared to nuclear DNA, mitochondrial DNA suffers much greater damage with aging (Hudson et al., 1998; Barja and Herrero, 2000; Hamilton et al., 2001). Mecocci et al. (1993) reported that the levels of 8-oxodG in brain mtDNA are increased up to 15-fold in humans aged 70 and above. Moreover, Hamilton et al. (2001) reported that the levels of 8-oxodG in nuclear DNA (brain, liver, heart, kidney, spleen) and mtDNA (brain, liver) are significantly increased in rodents (rat and mouse) with age; they also found a threefold greater increase in mtDNA than in nDNA.

The underlying mechanism for age-dependent increases in brain oxidative DNA lesions is not fully understood. However, recent studies investigating age-related regulation of neuronal DNA repair activity offer strong clues that age-dependent decline in BER activity may explain the well-established phenotype of oxidative damage accumulation with age. For example, in the brain of C57BL/6 mice, cellular BER activity is found to decrease about 50–75% with age (24-month old versus 4-month old), and the age-related reduction in repair capacity correlates with decreased levels of DNA polymerase-β activity, protein, and mRNA (Rao et al., 2001; Cabelof et al., 2002). In a study by Intano et al. (2003), an 85% decline in repair activity was observed in brain nuclear extracts prepared from old mice compared with young mice. An investigation of the effect of aging on neuronal BER activity found that mitochondrial repair activity showed marked age-dependent declines in the brain (Chen et al., 2002). The levels of overall BER activity were highest at E17, gradually decreased thereafter, and reached the lowest at the age of 30 months (∼80% reduction). The decline in overall mitochondrial BER activity with age was attributed to the decreased expression of repair enzymes such as 8-OHdG glycosylase and DNA polymerase-gamma and, consequently, the reduced activity at the steps of lesion-base incision, DNA repair synthesis and DNA ligation in the BER pathway (Chen et al., 2002). Interestingly, the age-dependent deficiency in neuronal DNA repair capability is also manifested in decreased adaptive responses of the brain to oxidative stress. In a study by Edwards et al. (1998), the brain level of the DNA repair enzyme APE was increased two- to threefold in young (3-month old) male rats exposed to isobaric hyperoxia, an oxidative stressor that stimulates DNA repair, whereas this adaptive response was lost in older (30-month old) rats.

Collectively, the decline in BER activity may be an important mechanism responsible for the age-dependent accumulation of oxidative DNA lesions in the brain and thus may contribute to the pathogenesis

of neurodegenerative diseases involving genomic instability and mutagenicity. According to this theory, an attractive approach to limiting endogenous oxidative DNA damage in the brain would be to reverse the age-dependent decline of BER activity. Caloric restriction has been a potent experimental manipulation found to reverse the age-dependent decline in nuclear DNA repair activity (Cabelof et al., 2003; Stuart et al., 2004). When rats or mice were subjected to caloric restriction to 60% of normal levels, their genomes were clearly protected from deleterious damage with age, and accompanied with this genomic protection was a complete reversal of age-related decline in BER capacity in all tissues tested (brain, liver, spleen, and testes) (Cabelof et al., 2003). These results shed light on potential new strategies to attenuate oxidative DNA damage during aging in the brain.

3.3 Regulation of the Base Excision Repair Pathway After Ischemia

Under certain circumstances, mammalian cells have the capacity to enhance their DNA repair activity in response to oxidative stress or genotoxicity (Herrlich et al., 1984; Nebert et al., 1990), employing a special form of adaptive cellular response reminiscent of the so-called SOS response originally characterized in bacteria. For example, treatment of Chinese hamster ovary (CHO) cells with sublethal levels of oxidative stressor dramatically increases the levels of APE expression and renders the cells more resistant to the clastogenic effect of subsequent challenge with lethal doses of oxidative stress (Grosch et al., 1998). In the human HeLa S3 tumor line and in WI 38 primary fibroblasts, the APE gene is also activated selectively by sublethal levels of a variety of ROS and ROS generators, which significantly increase the resistance of cells to the cytotoxicity of such ROS generators as H2O2 and bleomycin (Ramana et al., 1998).

How is the BER pathway regulated in the brain after cerebral ischemia? Studies suggest that several components of the BER pathway are inducible in the brain in response to ischemic challenges. The first evidence came from a study by Lin et al. (2000), which showed that the protein and activity of OGG1, the enzyme responsible for the repair of 8-oxodG, were significantly increased in the mouse brain after 90 min of forebrain ischemia and 20–30 min of reperfusion. The increase in the brain OGG1 correlated positively with the elevation of ischemia-induced DNA lesions in a reporter gene (the c-fos gene), suggesting that OGG1 may have a functional role in reducing oxidative gene damage in the brain after ischemia/reperfusion (Lin et al., 2000). A recent study by Lan et al. (2003) showed dynamically the inducible responses of nuclear BER in the rat model of MCA occlusion and reperfusion. The BER-mediated DNA repair synthesis, which represents the overall BER activity, was markedly (two- to fourfold) and persistently (up to 72 h) increased during reperfusion in the frontal/parietal cortex, the regions that survived ischemia in this model. The induced BER activity in nuclear extracts was attributed to the upregulation of gene expression and activation of selective BER enzymes, including DNA polymerase-β and OGG1. This induced BER activity in the ischemic cortex likely contributed to the efficient repair of several types of oxidative DNA lesions such as 8-oxodG, AP sites. and SSBs (Lan et al., 2003). In addition to the inducible BER response in the nuclear extracts, a similar inducible response involving multiple BER components was also observed in brain mitochondria after brief focal ischemia in the rat (Chen et al., 2003). Thus, in response to sublethal ischemic insults, the brain is able to activate the BER pathway in both nuclear and mitochondrial genomes. This inducible BER response following ischemia may represent an important mechanism underlying endogenous neuroprotection against oxidative neuronal injury by preventing the accumulation of cell-killing DNA lesions.

In contrast to the inducible response of BER to sublethal ischemia, BER activity is downregulated following a severe ischemic insult. As studied in the transient focal ischemia model, multiple components of the BER pathway were found to be persistently decreased in both nucleus and mitochondria in the ischemic core destined to infarct (Chen et al., 2003; Lan et al., 2003). The failure of BER in the ischemic core may play an important contributing role in the persistent accumulation of oxidative DNA lesions in this region (Chen et al., 2003). In contrast to the effect of inducible BER in the ischemic penumbra. Further evidence that failure in DNA repair may contribute to cell death after ischemia comes from studies showing the decreased expression of BER enzymes APE and X-ray repair cross-complementing group 1 (XRCC1) after severe cerebral ischemia, particularly in cell populations that eventually develop DNA fragmentation (Fujimura et al., 1999; Kawase et al., 1999).

The intracellular signaling mechanisms underlying the regulation of BER activity after ischemia have not been defined thus far. The induced activation of certain BER enzymes such as OGG1 and DNA polymerase-β may be related to gene transactivation at transcriptional levels. Several factors involved in ischemic injury, such as hypoxia and oxidative stress, could activate the transcriptional process of DNA polymerase-β by enhancing the DNA binding of ATF-1/CREB. ATF-1/CREB is the major promoter activator of the DNA polymerase-β gene (Narayan et al., 1996), and several recent studies have confirmed its upregulation in neurons that survived ischemia or oxidative stress (Zaman et al., 1999; Tanaka et al., 2000; Mabuchi et al., 2001). ATF-1/CREB is also a potent promoter activator of the APE gene in response to oxidative stress (Grosch and Kaina, 1999). The promoter of OGG1 has recently been studied, and the transcriptional factor nuclear factor-YA (NF-YA) was found to regulate the induction of human OGG1 following treatment with DNA-alkylating agent methylmethane sulfonate (MMS) in HCT116 cells (Lee et al., 2004). NF-YA is a general transcription factor that is activated by oxidative stress or ER stress and is involved in transcription of numerous genes (Maity and de Crombrugghe, 1998; Yoshida et al., 2001). An important objective for future studies will be to identify the transcriptional factors that regulate BER enzymes in ischemic neurons.

3.4 Base Excision Repair Activity and Cell Protection

Work in nonneuronal mammalian cells has defined a strong linkage between cellular BER capacity and the fate of cells that are subjected to oxidative stress or other genotoxic insults. At least three lines of evidence support a critical role of BER activity in promoting cell survival following genotoxic stresses and DNA damage. First, numerous studies demonstrate that defective repair of endogenous DNA lesions enhances vulnerability of cells to DNA damage (reviewed in Wilson and Thompson, 1997). For example, HeLa cells made deficient in APE activity by expression of an APE antisense construct become highly sensitive to a wide range of DNA-damaging agents, including hydrogen peroxide, paraquate, and hypoxic injury, and show increased cell death in response to DNA damage (Walker and Sikorska, 1994). Also, expression of a dominant negative rat β-pol mutant in cells interferes with BER and greatly enhances the cell-killing effect of DNA-damaging agents (Clairmont and Sweasy, 1996). Similar effects are seen in β-pol knockout mouse cells as well, where reduced BER activity is associated with increases in DNA damage-induced apoptosis (Sobol et al., 1996; Kaina et al., 1998; Ochs et al., 1999). Similarly, mouse cells deficient in the XRCC1 or PARP-1 gene show marked delay in DNA strand-break rejoining and decreased cell viability in response to DNA damage-inducers (Wilson and Thompson, 1997; Trucco et al., 1998; Tebbs et al., 1999).

The second line of evidence involves studies showing that mice carrying targeted disruption of a BER gene, β-pol, APE, or XRCC1, die in embryo at early or mid-gestation phase, and all show a defective neurogenesis (Sobol et al., 1996; Xanthoudakis et al., 1996; Wilson and Thompson, 1997; Tebbs et al., 1999; Sugo et al., 2000). Particularly well-studied in β-pol-knockout mice, a defective neurogenesis is characterized by extensive apoptosis occurring in the developing central and peripheral nervous systems, indicating that defective repair of endogenous DNA damage is destructive for neural development (Sugo et al., 2000).

Finally, a number of studies show that enhanced BER capacity renders cells more resistant to the cell-killing effect of DNA damage and oxidative stress. For example, when the expression of APE is induced in cells by sublethal levels of ROS or ROS generators, those cells become more resistant to the cytotoxicity of lethal doses of oxidative stress or other DNA-damaging agents (Grosch et al., 1998; Ramana et al., 1998). This effect has been seen in several types of mammalian cells including HeLa cells, WI38 human primary fibroblasts, and CHO cells. The protection can be attributed, at least in part, to the enhanced capacity for the repair of AP sites (Grosch et al., 1998; Ramana et al., 1998). Two studies suggest that upregulation of β-pol-dependent BER activity also offers protection in mouse fibroblasts and monocytes against DNA damage-induced cytotoxicity (Chen et al., 1998a; Horton et al., 2000), and this protection appears to be partially mediated by the activation of the long-patch BER pathway (Horton et al., 2000). Several studies using gene transfection approaches to enhance BER capacity in mammalian cells show protection of cells against oxidative stress (Tomicic et al., 1997; Grosch et al., 1998; Hansen et al., 1998). For example, transfection of human APE cDNA driven by an inducible promoter into CHO cells markedly reduced

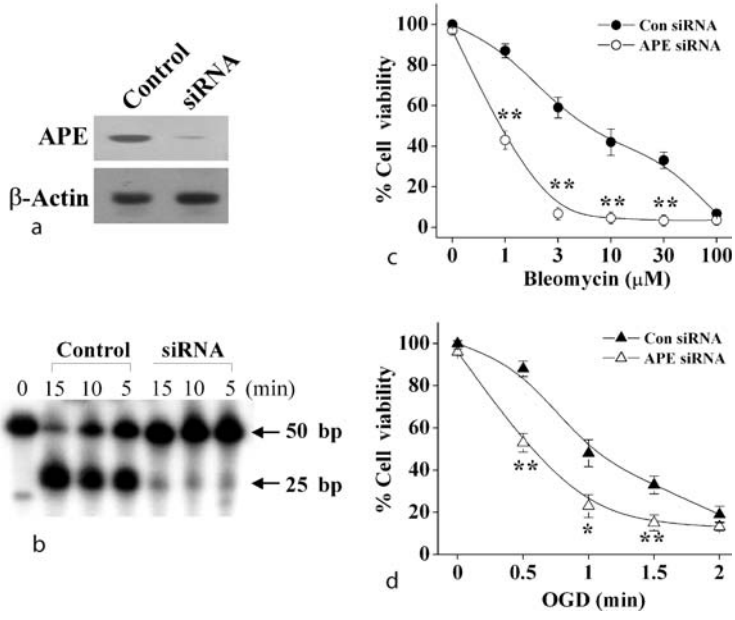
cytotoxicity of oxygen-derived free radicals (Grosch et al., 1998). Moreover, stable transfection in HeLa cells of a chimeric DNA repair protein created from APE and O6-methylguanine DNA methyltransferase (MGMT) protects against DNA damage-induced cytotoxicity (Hansen et al., 1998). Taken together, all of the findings described here support the notion that an alteration in DNA BER activity can substantially affect the fate of cells that are subjected to oxidative stress or other genotoxic insults.

3.5 The Role of Base Excision Repair Activity in Neuroprotection

We have just begun to understand the functional significance of regulation of neuronal BER activity in neuronal cells. In primary cultures of cortical neurons, several preconditioning paradigms, such as sublethal doses of sodium hypochlorous acid or bleomycin, can upregulate the expression and activity of APE and consequently boost the overall cellular BER activity (Li, Gobbel and Chen, unpublished data). When applied to neurons 16 h prior to the subsequent lethal insult, these preconditioning challenges reduced cell death by 40–80%, suggesting that preconditioning had activated endogenous neuroprotective mechanisms. To determine the potential role of APE-dependent BER activity in neuroprotection, we have manipulated the level of APE expression in neurons using siRNA and transfection-mediated overexpression strategies, respectively. Vector-mediated siRNA expression depleted over 90% of APE in neurons, causing the loss of AP site-cleavage activity in nuclear extracts (*Figure 5-4*). Suppression of APE expression resulted in strikingly increased sensitivity of neurons to the cytotoxic effect of bleomycin, a DNA damaging

Figure 5-4

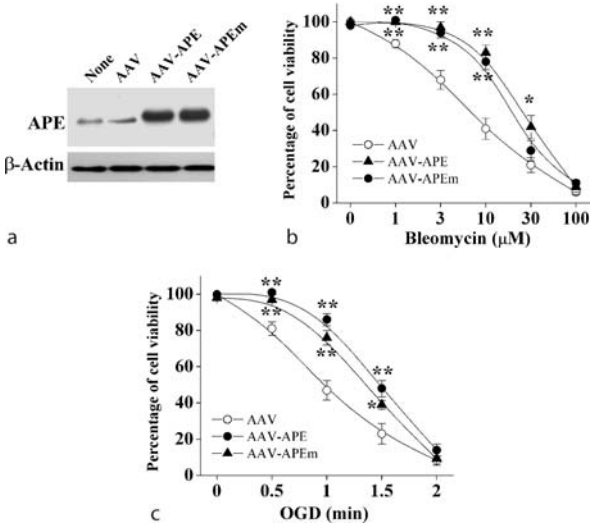
APE activity is essential for neuronal survival in response to oxidative injury. (a) AAV-mediated siRNA transfection depleted APE in primary cortical neurons. Western blot analysis was performed at 48 h after siRNA transfection. (b) siRNA transfection resulted in the loss of APE activity in neurons, as determined using the substrate-specific AP site-cleavage assay. The size of the specific cleavage product is 25 bp. (c and d) Depletion of APE activity in neurons diminished cell survival in cultures in response to oxidative injury induced by Bleomycin or OGD. Cell viability was measured at 24 h after injury using the LIVE/DEAD viability/cytotoxicity kit (Molecular Probes). Data are mean ± SEM, $n = 9$ per data point from two to three independent experiments. *$p < 0.05$; **$p < 0.01$ versus control siRNA-transfected cells



agent, and oxygen-glucose deprivation that simulates ischemic injury in vitro (*Figure 5-4*). In contrast to the effect of APE gene knockdown, overexpression of APE in neurons via adeno-associated virus (AAV) vector-mediated gene transfection boosted the APE-dependent BER activity and significantly protected against cell death induced by bleomycin or oxygen-glucose deprivation (*Figure 5-5*), but not by UV (Li,

Figure 5-5

Enhanced APE activity increases neuronal survival after injury. (a) AAV-mediated overexpression of the wild type APE or mutant (Cys65Ala) AP endonuclease (APEm) lacking the redox-regulation activity in primary cortical neurons. (b and c) Graphs show the protective effect of APE overexpression against Bleomycin or OGD induced cell death in cultured neurons. Cell viability was measured at 24 h after injury using the LIVE/DEAD viability/cytotoxicity kit (Molecular Probes). Data are mean ± SEM, $n = 12$ per data point from three independent experiments. *$p < 0.05$; **$p < 0.01$ versus empty AAV-transfected cells



Gobbel and Chen, unpublished data). These results suggest that induced BER activity was responsible for the protection, as UV-induced DNA damage is repaired by the NER pathway but not by BER. Interestingly, the APE mutant lacking the redox function also showed significant protection in transfected neurons, indicating that the redox-regulation activity may not be essential for APE's protective effect. These results provide evidence that alteration of BER activity via the regulation of a key repair enzyme substantially affects the outcome of neuronal injury in primary cultures induced by DNA damage, oxidative stress, or ischemia.

A recent report by Endres et al. (2004) offers the first direct evidence that components of the BER pathway play a protective role against ischemic brain injury. Mice deficient in uracil-DNA glycosylase (UNG), the enzyme involved in BER of aberrant uracil residues in nuclear and mitochondrial DNA, were subjected to 30 min of transient MCA occlusion. Infarct sizes, as determined at 72 h after ischemia, were markedly increased (>twofold) in *Ung*(–/–) mice as compared to their wild-type littermates. After combined oxygen–glucose deprivation, *Ung*(–/–) primary cortical neurons derived from the knockout mice also showed increased vulnerability to cell death, which was associated with early mitochondrial dysfunction (Endres et al., 2004). Therefore, UNG-dependent BER activity seems to be critical for neuronal survival in the brain after ischemic challenges.

4 Conclusions and Emerging Hypotheses

A growing body of experimental evidence emphasizes the importance of the accumulation of oxidative DNA damage in the pathophysiology of ischemic brain injury. Unrepaired oxidative DNA damage is an

important trigger of neuronal apoptosis and necrosis. Thus, a fully functional DNA repair system is essential for neurons to survive, especially under ischemic conditions when there is increasing demand for DNA repair. As the predominant repair mechanism for oxidative DNA lesions in the brain, the BER pathway is highly inducible in response to sublethal ischemic, hypoxic, or oxidative stress challenges, and the induced BER activity in cells has been associated with rapid repair of endogenous DNA damage and cell survival. Accordingly, it has been speculated that BER is a novel inducible mechanism for neuroprotection in the brain. One attractive hypothesis is that neuroprotection against ischemic neuronal injury may be achieved by enhancing cellular BER activity. This hypothesis has now been partially addressed in neuronal cultures using gene transfection of BER repair enzymes. However, a more substantial question remaining to be addressed is whether pharmacological or molecular strategies that induce BER activity in vivo can offer neuroprotection against ischemic brain injury.

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6 Heat-Shock Protein Regulation of Protein Folding, Protein Degradation, Protein Function, and Apoptosis

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Abstract: Heat-shock proteins (Hsps), induced by heat and other stresses via heat-shock factor (HSF) actions on heat-shock elements (HSEs) in Hsp promoters, bind to and regulate other proteins. Hsc70 regulates folding while proteins are being synthesized on the ribosome and chaperones proteins to intracellular organelles. Hsp70, in combination with cochaperones Hip and Hop, refolds partially denatured proteins. Hsp70, in combination with cochaperones BAG-1 and CHIP, targets proteins for degradation in the proteasome. Hsp90 chaperones proteins—including HSF, estrogen receptor, and NOS—to regulate their availability and function. The mitochondrial Hsp70 (mtHsp70), together with cochaperones, facilitates protein/peptide entry into mitochondria. Hsps also play major roles in regulating apoptosis. Hsp70 modulates mitochondrial-caspase-3-dependent apoptosis by binding apoptotic protease activation factor-1 (Apaf-1) and disrupting the formation of the Apaf-1/cytochrome *c*/caspase-9 apoptosome, which prevents activation of caspase-3. Hsp70 binds AIF to prevent mitochondrial-mediated, caspase-independent apoptosis. JNK phosphorylation and acts upstream of the mitochondria to prevent stress-mediated mitochondrial-induced apoptosis. The expression of Hsp70 and other Hsps is generally protective as Hsp70 transgenic mice and viral overexpression of Hsps protect neurons, brain, and cells against ischemia and other types of injury. However, Hsp70 facilitates TNF-mediated cell death via the extrinsic apoptotic pathway by binding IKKγ and preventing activation of NF-κB signaling that promotes caspase-8 activation of caspase-3 and leads to cell death. Coincident TNF receptor activation and Hsp70 expression is lethal to the cell. The mtHsp60 and Hsp10 protect against protein aggregation and refold denatured proteins; but when released from mitochondria also activate caspase-3 and produce apoptosis. Thus, Hsps promote proper protein folding and protein refolding, disaggregate proteins and chaperone proteins across membranes; or they can target proteins for degradation and even stimulate cell apoptosis.

1 Introduction

Heat-shock proteins (Hsps) are a family of proteins that are induced by heat and many other stresses including ischemia, heavy metals, excitoxicity, hypoglycemia, and a wide variety of acquired and hereditary neurological diseases. Initially identified by chromosome puffs when flies were heated, the proteins were defined as being heat inducible and are now defined as having heat-shock elements (HSEs) in their promoters (Lindquist, 1986, 1992).

The main members of the family include Hsp100/104, Hsp90, Hsp70/Hsc70, Hsp60 and Hsp10, mitochondrial Hsp70 (mtHsp70), Hsp40, Hsp32 [heme oxygenase-1 (HO-1)], Hsp25/27, and other small Hsps including α-crystallin. The high molecular weight Hsps are ATP dependent, whereas the lower molecular weight members are ATP-independent chaperones (Parcellier et al., 2003). A large number of other molecules have been discovered that act as cochaperones for these and other Hsps including Hip, Hop, BAG-1, CHIP, auxilin, GAK, CSP, SGT, Djp1, Tom 70, Tom34, UNC-46, FKBP52, Cyp40, Mrj, SODD/BAG-4, CAIR-1/BAG-3, and Snl1 (Young et al., 2003). Hsps play a role in moving proteins across membranes and into all cellular compartments, folding of proteins in all cellular compartments, degradation of unstable proteins, dissolving protein complexes and protein aggregates, preventing protein aggregation, controlling regulatory proteins, and refolding of misfolded proteins (Garrido et al., 2001; Parcellier et al., 2003).

As described in detail later, Hsp100 family members play role in disaggregating protein aggregates in concert with Hsp70/Hsp90/Hsp40. Hsc70 is constitutively expressed in all cells, binds to newly synthesized proteins on the ribosome, and probably prevents abnormal folding until the proteins are released at their intracellular targets. Hsp90 binds a large number of substrates and limits their availability and thereby regulates their function within the cell. Hsp70 is induced in response to denatured proteins and together with Hsp40, Hsp90, and Hip and Hop serves to renature proteins within the cell. MtHsp70 chaperones proteins across mitochondrial membranes. Hsp60 and Hsp10 help to form protein complexes within the mitochondria and also renature partially denatured proteins within the mitochondria. Hsp32/HO acts as an enzyme to oxidize heme to biliverdin within cells and may help to chaperone heme. Hsp25 binds actin

and likely helps shape and reshape stressed cells. The small Hsps including α-crystalline help to either form large protein aggregates or prevent denatured aggregates from forming (Young et al., 2003).

Because Hsps are intimately involved with virtually all aspects of protein function and cell signaling, they are also being increasingly recognized as playing central roles in virtually all types of acute and chronic neurological diseases. Hsps are induced in the brain following stroke, hypoglycemia, seizures, and trauma and serve to prevent protein denaturation and help to renature proteins and protect cells from a wide variety of stresses. In chronic neurodegenerative diseases in which mutations and aging lead to misfolded proteins, this results in formation of cellular inclusions that are recognized as collections of misfolded proteins and that are often neurotoxic and the proximal causes of the cell death and neurological symptoms.

2 Role of Hsp70 in Regulating Protein Folding and Protein Degradation

2.1 Hsp70 Regulation of Protein Folding

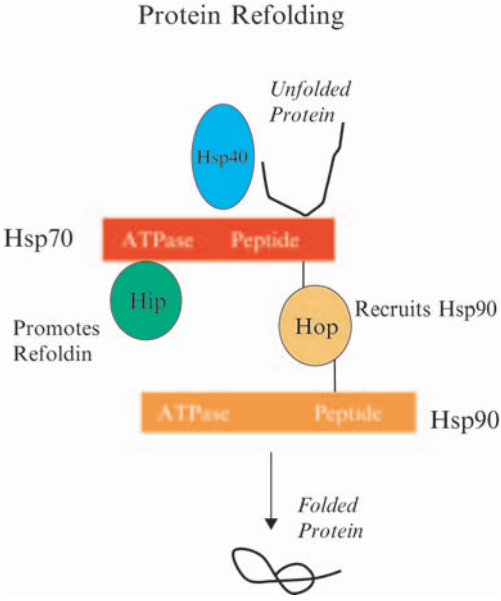
Hsps were initially defined by their induction by heat. It has since been learned that heat shock activates transcription factors called heat-shock factors (HSFs). HSFs are normally bound by Hsp90 in an inactive state (see later). Apparently, once denatured proteins appear in a cell, these malformed proteins are recognized and bound by Hsp90. This results in the release of HSFs that then can bind to HSEs in the promoter of heat-shock genes (Lindquist, 1986). HSF binding to HSEs in the promoters of the Hsps initiates transcription of the heat-shock genes including various Hsp families, including Hsp100s, Hsp90, Hsp70s, Hsp60, Hsp32, Hsp10, and other small Hsps.

Hsp70, in concert with other chaperones, participates in the folding of newly synthesized proteins, protection of proteins during periods of cellular stress, and plays a role in intracellular protein trafficking (Lindquist, 1986; Hohfeld et al., 2001). Hsp70, along with Hsp90, associates with nonnative protein substrates through recognition of hydrophobic patches buried in the native structure. This interaction is coupled to cycles of ATP binding and ATP hydrolysis by the chaperones, with substrates being released when ATP binding occurs to the N terminus. When the proteins are released, further folding or other protein modifications can occur after which the chaperones may rebind and continue to refold (Hohfeld et al., 2001).

Hsp70, as well as Hsp90, has an ATPase N-terminal binding domain and a peptide binding C-terminal domain. Proteins that interact with Hsp70, as cochaperones, generally act at the N terminus and can have two primary modes of action. They can either regulate the ATPase cycle of the chaperone to influence its affinity for protein substrates or recruit the chaperones to specific proteins, protein complexes, and subcellular compartments (Hohfeld et al., 2001). A chaperone-binding motif found in several Hsp70 and Hsp90 cofactors is characterized by a tandem arrangement of three degenerate 34-amino acid repeats (tetratricopeptide repeats, TPRs) (Frydman and Hohfeld, 1997). The Hsp70/Hsp90-organizing protein Hop possesses multiple TPR domains, enabling it to simultaneously bind Hsp70 and Hsp90 and to promote chaperone cooperation for protein refolding or regulation of protein function (Frydman and Hohfeld, 1997). Hop binds preferentially to the ADP-bound form of Hsp70 (Pratt and Toft, 2003). Hip binds to the ATPase domain of Hsp70, stabilizing the ADP state, which has a high affinity for nonnative substrate protein. Hip and BAG-1 compete with each other in binding to the Hsp70 ATPase domain (Hohfeld et al., 2001; Pratt and Toft, 2003). Thus Hip and Hop, together with Hsp40, and binding to Hsp90, form the Hsp70 folding machine (*Figure 6-1*) (Hohfeld et al., 2001). Hip binding promotes refolding by Hsp70; and Hop binding to the C terminus of Hsp70 recruits Hsp90 which is a requisite partner for Hsp70 to efficiently refold target substrate proteins (Frydman and Hohfeld, 1997; Bukau and Horwich, 1998; Hohfeld et al., 2001).

Hsp70 stabilizes partially unfolded/denatured proteins, resulting in higher efficiency of refolding or in prevention of aggregation. Hsp70, at least by itself, probably does not disaggregate large protein aggregates (Voos and Rottgers, 2002).

Figure 6-1
Hsp70 protein, in collaboration with cochaperones Hsp40, Hip, Hop and Hsp90, plays a central role in folding unfolded proteins and in refolding partially denatured proteins. Modified from Hofeld et al. (2001)



2.2 Hsp70 Regulation of Protein Degradation

Hsp70 can also be involved in protein degradation that is dependent on which cochaperones bind to it (Wickner et al., 1999; Hohfeld et al., 2001; Cyr et al., 2002). CHIP has a single TPR domain that can contact either Hsp70 or Hsp90. Association of CHIP with Hsp70 blocks the ATPase cycle of the chaperone and inhibits its ability to refold nonnative proteins. CHIP has a similar effect on Hsp90 and therefore impairs the ability of both chaperones to assist cellular protein folding.

The C terminus of CHIP has structural similarities to components of the ubiquitin/proteasome system (Hohfeld et al., 2001; Cyr et al., 2002). Indeed, CHIP has been shown to be a ubiquitin ligase and is the first such protein to directly bind known chaperones (Cyr et al., 2002). It appears that CHIP and Hop compete for the C terminus binding to Hsp70 and Hsp90. If CHIP binding predominates, then the protein substrate bound to the Hsp70/Hsp90 protein-folding machine is targeted to the proteasome (*Figure 6-2*). If Hop binding predominates, the protein is refolded (*Figure 6-1*).

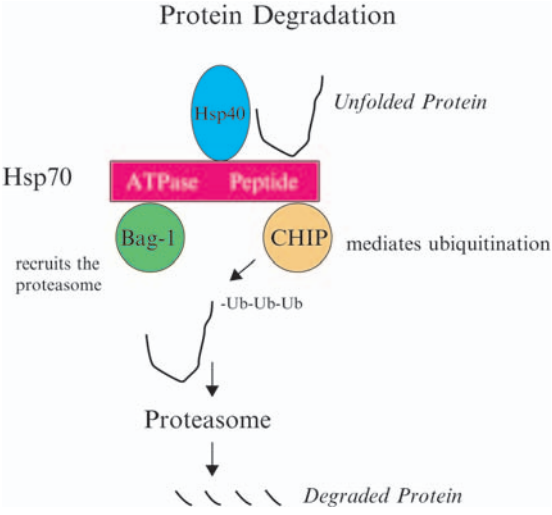
BAG-1 has also been proposed to act as a link between molecular chaperones and the ubiquitin/proteasome system (Luders et al., 2000). The N terminus interacts with the proteasome and the C terminus with Hsp70. BAG-1 binds to the ATPase domain of Hsp70 and promotes the release of ADP (Hohfeld et al., 2001; Cyr et al., 2002).

Of note, the different Hsps appear to target different proteins for degradation. Hsc70 is necessary for the degradation of actin, α-crystallin, glyceraldehydes-3-phosphate dehydrogenase, β-lactalbumin, and histone H2A, while it is not required for the degradation of bovine serum albumin, lysozyme, and oxidized RNase A. Hsp70 interactions with CHIP and Hsp90 favor degradation of CFTR, Parkin, and the glucocorticoid receptor. Hsp27 facilitates I-kBα degradation but not p27 or cyclin E.

2.3 Heat-Shock Proteins, Protein Degradation, and Protein Aggregates

As Hsp70 and many of the other Hsps are involved in folding proteins as well as in protein degradation, it is not surprising that they might be involved in producing protein aggregates and in dissolving protein

Figure 6-2
Hsp70, in collaboration with cochaperones, including Hsp40, BAG-1, and CHIP, serves to target some substrate proteins for ubiquitnation and degradation in the proteasome—if they cannot be folded or refolded. Modified from Hofeld et al. (2001)



aggregates. Proteasome inhibitors trigger the formation of aggregates in cells and eventually lead to cell death by apoptosis (Garrido and Solary, 2003). Both Hsp70 and small Hsps are induced by disruption of the proteasome and may attempt to delay death.

The Hsp100 family of Hsp (including Hsp104 in yeast, ClpB, Hsp101, and Hsp78), also named Clp proteins, form large homo-oligomeric protein complexes, usually rings of six or seven subunits. Hsp100 proteins appear to play a major role in resolubilization of large protein aggregates (Glover and Lindquist, 1998; Voos and Rottgers, 2002; Mogk and Bukau, 2004). Hsp100 family members in bacteria prevent cellular damage caused by the accumulation of aggregated proteins. The bacterial ClpB and ClpA dissolve protein aggregates in an ATP-dependent reaction and make smaller aggregates. Hsp100 proteins, with the help of the Hsp70 system, disaggregate large complexes into smaller ones and help to unfold proteins so that they can be refolded by the Hsp70/Hsp90 machine to their native conformations and restore protein function (Glover and Lindquist, 1998; Mogk and Bukau, 2004). The Hsp100 disaggregator appears to physically associate with the Hsp70 unfolder/folder machine (Mogk and Bukau, 2004). Hsp100 family members also associate with specific proteases that mediate protein turnover (Voos and Rottgers, 2002). Hsp100 family members may also play a direct role in protein degradation. Cochaperones either drive Hsp100s to disaggregate proteins and refold them or disaggregate proteins and degrade them in the proteasome and lyosomes (Voos and Rottgers, 2002) similar to the model for Hsp70 shown earlier (*Figures 6-1* and *6-2*).

Hsp104 also appears to play a role in prion formation in cells (Shorter and Lindquist, 2004). Hsp104 regulates inheritance of [PSI+], a yeast prion formed by self-perpetuating amyloid forms of the translation termination factor Sup 35. Low concentrations of Hsp104 catalyzed the formation of oligomeric intermediates that proved critical for the nucleation of Sup 35 fibrillization and displayed a conformation common among amyloidogenic polypeptides (Shorter and Lindquist, 2004). At higher Hsp104 concentrations, amyloidogenic oligomerization and contingent fibrillization were abolished. Hsp104 also disassembled mature fibers in a manner that initially exposed new surfaces for conformational replication but eventually exterminated prion conformers. These Hsp104 activities differed in their reaction mechanism and explained [PSI+] inheritance patterns (Shorter and Lindquist, 2004). These and other studies suggest that regulation of normal cellular prion protein and its conversion to self-perpetuating, disease-producing prion protein may be regulated by chaperones.

Hsp27 and α-crystalline also appear to associate with the proteasome (Garrido and Solary, 2003). Hsp27 directly interacts with ubiquitin, which may account for colocalization of Hsp27 with ubiquitinated proteins and the 20S proteasome in cytoplasmic inclusions found in a variety of degenerative diseases (Parcellier et al., 2003). Thus like Hsp70, the small Hsps probably play a role not only in protein folding and refolding but also in targeting proteins—that presumably cannot be refolded—to the proteasome and other proteolytic sites in the cell (Garrido and Solary, 2003).

3 Chaperone Functions of Heat-Shock Proteins

A number of Hsps function mainly as chaperones for other proteins. This chaperone function is characterized by binding to target proteins and then releasing those proteins. The proteins that are targeted are often determined by cochaperones that bind the primary chaperone. In addition, the intracellular target or intracellular organelle target are again modulated by cochaperones that bind the primary chaperone. Although there are many chaperones in cells, three main chaperones will be reviewed here: Hsc70; Hsp90; and the mtHsp70. Each chaperone has unique functions that vary depending on the binding of cochaperones.

3.1 Hsc70: The Constitutive Heat-Shock Protein 70

Hsc70 is constitutively expressed in virtually every known cell from plants to primates. Hsc70 binds to peptides/proteins as they are being formed on the ribosome and probably prevent premature folding of the partially formed protein (Beckmann et al., 1990). In addition, once fully formed, Hsc70 chaperones the newly formed protein to its target site in the cell and help translocate proteins across membranes in the ER, lysosome, mitochondria, and peroxisomal membranes. The association of Hsc70 with specific cochaperones might explain how proteins are targeted to specific organelles (Young et al., 2003). Djp1 binding to Hsc70 may target proteins to peroxisomes, and Tom70 and Tom34 binding to Hsc70 together with Hsp90 may target proteins to the mitochondria. Of note, Hsp90-binding drugs like geldanamycin block protein import into mitochondria. Once at the target organelle, BAG-1 interacts with the ATPase domain of Hsc70 and triggers the exchange of ADP for ATP, favoring the release of Hsc70-bound peptides (Young et al., 2003).

Hsc70 also plays a central role in uncoating of clathrin-coated vesicles. The clathrin-binding domain of auxilin assembles onto clathrin cages, and the J domain of auxilin stimulates free Hsc70 to hydrolyze ATP. Hsc70 in the ADP state binds to clathrin and distorts the conformation of clathrin leading to disassembly of the clathrin cage. Clathrin can then recycle back onto membranes (Young et al., 2003). Disruption of Hsc70 function causes defects in endocytosis and in vesicle transport between the trans-Golgi, endosomes, and plasma membrane. Hsc70 together with cysteine string protein (CSP) and some SNARE proteins are involved in exocytosis as well, which appears to be independent of clathrin (Young et al., 2003). The ATPase activity of Hsc70 in turn is regulated by CSP and small glutamine-rich TPR protein (SGT) (TPR domain).

Hsc70 appears to specifically regulate the neuronal Rab3A protein, which is a GTPase that limits the extent of calcium-stimulated exocytosis. GTP hydrolysis by Rab3A on vesicle membranes is activated upon vesicle fusion, and the aGDI protein transfers Tab3A from the membrane to the cytosol. aGDI binds Rab3A on the membrane in a complex with CSP, Hsc70, and Hsp90. The binding of aGDI by Hsp90 and Hsc70 is proposed to facilitate the extraction of Rab3A and its reassembly on the membrane in the GTP-bound state (Young et al., 2003).

Hsc70 also binds the cochaperone Mrj that specifically recognizes the keratin 18 component of keratins 8 and 18. The Hsc70 recruited by Mrj thus acts in the regulated assembly of the keratin filament cytoskeleton (Young et al., 2003).

The BAG-1 related family of proteins are nucleotide exchange factors for Hsc70 that probably stimulate ATP cycling by Hsc70 rather than stabilizing its binding to polypeptides. SODD or BAG-4 associates with

the cytosolic region of TNFR1 and BAG-3 associates with PLCγ. BAG-4 binds inactive TNFR1 and BAG-3 binds inactive PLCγ and are released when TNF binds TNFR1 or when PLCγ is phosphorylated by EGF-R (Young et al., 2003). Hsc70 is bound to the BAG proteins and may play a role in the conformational changes required for the switching.

3.2 Hsp90

Hsp90 is one of the most abundant proteins in unstressed cells (1%–2% of cytosolic protein) and it binds nearly 100 proteins and thereby regulates their functions. Hsp90 appears to interact with normally folded proteins and with intermediately folded proteins and to prevent their aggregation, but lacks the ability of Hsp70 to refold proteins. Hsp90 is highly efficient at preventing protein misfolding (Nollen and Morimoto, 2002). Hsp90 binds client proteins at chaperone sites and binds cofactors at other sites along with immunophilins that connect assembled substrate–Hsp90 complexes to protein-trafficking systems (Pratt and Toft, 2003). ATP hydrolysis by an intrinsic ATPase activity results in a conformational change in Hsp90 that is required to induce conformational change in a substrate protein. The conformational change induced in steroid receptors is an opening of the steroid-binding cleft so that receptor can be bound by steroid. Five proteins—Hsp90, Hsp70, Hop, Hsp40, and p23—are the minimum number of proteins required to assemble stable receptor–Hsp90 heterocomplexes (Pratt and Toft, 2003). An Hs90–Hop–Hsc70–Hsp40 complex opens the cleft in an ATP-dependent process. This produces a receptor–Hsp90 complex with bound ATP, following which p23 binds the complex to stabilize it. Hsp70 and Hsp40 first interact with the receptor in an ATP-dependent reaction. This complex is activated to a steroid-binding state in a second ATP-dependent reaction with Hsp90, Hop, and p23. This process occurs whether the substrate is a receptor, a protein kinase, or a transcription factor (Pratt and Toft, 2003). Hop binds an N-terminal TPR domain of Hsp70 and a central TPR domain of Hsp90, with Hsp90 functioning as a dimer (Pratt and Toft, 2003).

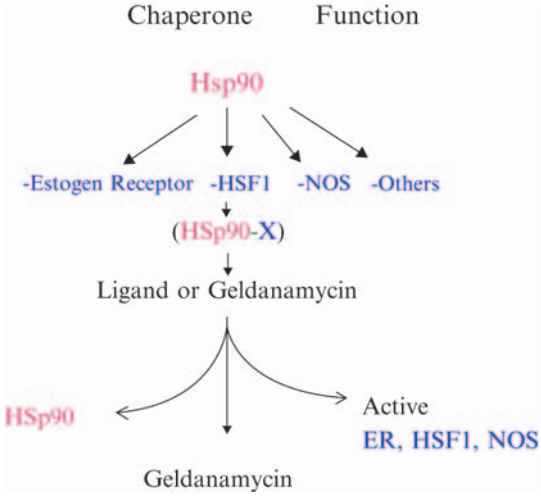
Examples of transcription factors and polymerases that are substrates for Hsp90 include: glucocorticoid receptor; progesterone receptor; estrogen and androgen receptors; mineralocorticoid receptor; retinoid receptor; HSF; heme activator protein; HIF-1α; telomerase; and SV40 large T antigen. Examples of signaling protein kinases that are substrates for Hsp90 and bind to it include: v-Src; v-yes; Sevenless PTFk casein kinase II, MEK; insulin receptor; insulin-like growth factor receptor; EGFR; c-Mos; Cdcks; MAK and MAK-related kinase; Akt kinase; and others. Other proteins bound at the chaperone site on Hsp90 include NOS, PAR-1, CFTR, ANPR; Apaf-1; Proteasome; lysosome; actin; tubulin; apoprotein B; and others (Pratt and Toft, 2003). Hsp90 (and perhaps Hsp27) appears to bind Akt and maintain its activity and thus promote survival by phosphorylating such proteins as Bad, forkhead, and IκB (Beere, 2004).

Proteins that bind at sites other than substrate sites include Hsp70, Hsc70, Hop, Dpit47, FKBP52, FKBP51, Cyp-40, PP5, ARA9, CHIP, UNC-45, p50, Harc, and calcium calmodulin (Pratt and Toft, 2003). Unc-45 isoforms are required for the correct assembly of conventional myosin into muscle thick filaments, which then contact the actin-based thin filaments of the sarcomere. Hsp90 recruited by Unc-45 probably aids in the folding and assembly and disassembly of myosin complexes. FKBP52 and cyclophilin Cyp40 bind Hsp90 that then interacts with dynein, a motor protein that moves along microtubules (Young et al., 2003). Thus, like Hsc70, it is likely that the binding of cochaperones to Hsp90 targets Hsp90 for certain substrates or classes of substrates or targets Hsp90 to certain organelles (Pratt and Toft, 2003). Binding of immunophilins to Hsp90 regulates the trafficking of steroid receptors between the cytoplasm and the nucleus (Pratt and Toft, 2003).

The N terminus of Hsp90 is the ATP binding site, and the site at which Hsp90 inhibitors geldanamycin and radicicol act. Geldanamycin binding to Hsp90, for example, releases HSFs that then act on HSEs in heat-shock genes to induce transcription of Hsp70 and other Hsps (Ali et al., 1998; Zou et al., 1998; Lu et al., 2002a). Hsp90 is essential for binding of heme by neuronal NOS (Pratt and Toft, 2003). The TPR-domain acceptor site on the Hsp90 dimer allows it to bind immunophilins and other proteins that bind to motor proteins and thus facilitate movement along cytoskeletal highways and from nucleus and back, thus helping large cells to function better (*Figure 6-3*).

Figure 6-3

Heat-shock protein 90 (Hsp90) binds to over 100 client proteins including the estrogen receptor, heat-shock factor 1, NOS isoenzymes, and many others. When the complex encounters the ligand, e.g., estrogen, or it encounters a Hsp90-binding drug like geldanamycin, Hsp90 facilitates conformational changes in the client protein so that it can bind the ligand or geldanamycin. Once bound, Hsp90 is released as is the ligand or geldanamycin. The client protein is released, generally in an active state



3.3 Mitochondrial Heat-Shock Proteins

Mitochondria contain several Hsps that are crucial for maintaining mitochondrial function. The mtHsp70 is essential for the translocation of precursor proteins in the cytoplasm across the two mitochondrial membranes (Voos and Rottgers, 2002). MtHsp70 interacts with preproteins in transit in an ATP-dependent reaction as they emerge from the translocation channel of the inner membrane. The ATPase domain of mtHsp70 is required for the translocation of proteins into mitochondria as is hydrolysis of ATP. MtHsp70, together with two essential partner proteins Tim44 and Mge1, forms a membrane-associated complex that unfolds preprotein domains and moves them across the mitochondrial membranes (Voos and Rottgers, 2002). Tim44 is an inner membrane anchor for soluble mtHsp70. Once proteins are in the matrix, the proper folding of the newly imported proteins is assisted by the soluble chaperone system formed by mtHsp70 and its partner protein Mdj1. MtHsp70 also plays a prominent role in the biosynthesis of mitochondrially encoded proteins via Mdj1. Mdj1 itself influences activity of the mitochondrial DNA polymerase. After being released by mtHsp70, the Hsp60 and Hsp10 mitochondrial Hsps assist in the proper folding for specific, newly imported proteins within the mitochondrial matrix (Voos and Rottgers, 2002). Hsp60 substrates are generally folding intermediates that have not acquired their native structure. Hsp60 acts in concert with two Hsp10 cochapersones and specific cyclophilins. The mtHsp70 Ssq1 is involved in the assembly of mitochondrial Fe/S clusters together with another member of the DnaJ family, Jac1. Chaperones of the Clp/Hsp100 family mediate the prevention of aggregation under stress conditions and eventually are involved in the degradation of mitochondrial proteins. Together, the chaperones of the mitochondrial matrix form a network that is essential for formation and degradation of most mitochondrial protein complexes (Voos and Rottgers, 2002).

Hsp78 is an Hsp100 family homolog found in mitochondria. Hsp78 appears to play an important role under severe periods of thermal or other cellular stresses. It may act on large protein aggregates to disaggregate them, and then in concert with mtHsp70 refold these proteins and restore their function (Voos and Rottgers, 2002). Similar mitochondrial proteins probably with similar functions have been identified in yeast (Mcx1) and in mammalian mitochondria (ClpP).

4 Apoptotic Pathways

There are at least three pathways that lead to apoptosis. One is the caspase-dependent mitochondrial intrinsic pathway. The second is a caspase-independent, mitochondrial-dependent pathway. The third is a caspase-dependent death receptor extrinsic pathway (Garrido et al., 2001).

In response to a number of proapoptotic pathways, the outer mitochondrial membrane becomes permeabilized, resulting in release of molecules normally confined to the intermembrane space. Such proteins move from the mitochondria to cytosol. Pore formation in the external membrane occurs via Bax, other related proteins, and physical disruption. The intrinsic pathway is activated by the release of cytochrome *c* through these pores. Cytochrome *c* interacts with apoptotic protease activation factor-1 (Apaf-1), triggering ATP-dependent oligomerization of Apaf-1 and exposing its caspase recruitment domain (CARD). Apaf-1 oligomer then binds to the CARD domain in procaspase-9 that leads to formation of the apoptosome. Activated caspase-9 cleaves procaspase-3. The activated caspase-3 acts on a variety of substrates to execute the apoptotic cell death program (Garrido et al., 2001).

Other proteins released from the mitochondria include the flavoprotein, apoptosis-inducing factor (AIF) and second mitochondrial-derived activator of caspase (Smac, also called Diablo). AIF released from mitochondria enters the nucleus and activates a DNAase to cleave DNA into large fragments (Garrido et al., 2001). Smac is also released by the permeabilized mitochondria. Smac activates apoptosis by neutralizing inhibitory activity of inhibitory apoptotic proteins (IAPs) that associate with and inhibit cysteinyl, *asp*artate-specific prote*ases* (caspases).

The extrinsic pathway involves plasma membrane death receptors. TNFR1, CD95, Fas, TRAILR1, DR3, and others that are members of the TNF superfamily of receptors that mediate inflammatory and immune responses (Garrido et al., 2001). Death receptors contain a death domain that interacts with the DD of cytosolic proteins TRADD or FADD that in turn interact with caspase-8. Caspase-8 (and perhaps caspase-10) can cleave procaspase-3 and activate executioner apoptotic pathways. In addition, caspase-8 cleaves Bid to truncated Bid (tBid) that acts on mitochondria to promote permeabilization and mitochondrial-cytochrome *c*-caspase-9, caspase-3-mediated apoptosis (Garrido et al., 2001).

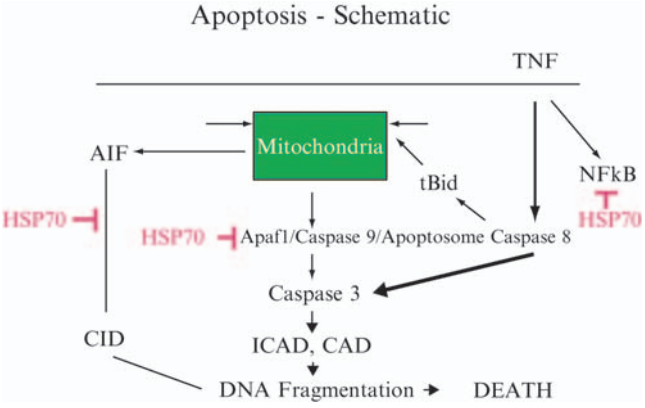
Caspases are the primary machinery by which apoptosis occurs. Initiator caspases interact with specific adaptor molecules to facilitate their own autocatalytic processing. These caspases (e.g., caspase-8, -9) in turn cleave and activate the downstream executioner caspases (caspase-3) that target proteins in the cell and kill it (Beere, 2004). Caspase-3 targets many proteins including inhibitor of caspase-activated DNAase (ICAD), caspase-activated DNAase (CAD), actin, subunits of mitochondria, and PARP (Beere, 2004) (*Figure 6-4*).

4.1 Hsp70 Regulation of Caspase-Independent Apoptosis

AIF is a phylogenetically ancient mitochondrial intermembrane flavoprotein endowed with the unique capacity to induce caspase-independent peripheral chromatin condensation and large-scale DNA fragmentation when added to purified nuclei (Cande et al., 2002). In addition to its apoptogenic activity on nuclei, AIF can also participate in the regulation of apoptotic mitochondrial membrane permeabilization and exhibits an NADH oxidase activity. Under normal circumstances, AIF is secluded behind the outer mitochondrial membrane. However, on apoptosis induction AIF translocates to the cytosol and the nucleus (Cande et al., 2002). Injection of anti-AIF antibodies or knockout of the AIF gene has demonstrated that AIF may be required for cell death occurring in response to some stimuli. Hsp70 protects Apaf-1(−/−) cells against death induced by serum withdrawal, indicating that Apaf-1 is not the only target of the antiapoptotic action of Hsp70 (Ravagnan et al., 2001). In a cell-free system, Hsp70 prevented the AIF-induced chromatin condensation of purified nuclei. Hsp70 specifically interacted with AIF, as shown by ligand blots and coimmunoprecipitation. Cells overexpressing Hsp70 were protected against the apoptogenic effects of AIF targeted to the extramitochondrial compartment (Ravagnan et al., 2001). In contrast, an antisense Hsp70 complementary DNA, which reduced the expression of endogenous Hsp70, increased sensitivity to the lethal effect of AIF. The ATP-binding domain (ABD) of Hsp70 seemed to be dispensable

Figure 6-4

Hsp70 binds AIF and inhibits AIF caspase-independent large fragment DNA fragmentation via a caspase-independent DNAase (CID). Hsp70 also binds Apaf-1 and prevents formation of the apoptosome and activation of caspase-3 and DNA fragmentation via a CAD. Hsp70 expression coincident with TNF activation of TNF receptors binds IKKγ, preventing NF-κB activation and promotes cell death via caspase-8 and caspase-3 pathways



for inhibiting cell death induced by serum withdrawal, AIF binding and AIF inhibition, although it was required for Apaf-1 binding (Ravagnan et al., 2001).

Inactivation of AIF renders embryonic stem cells resistant to cell death following growth factor withdrawal. Moreover, AIF is essential for programmed cell death during cavitation of embryoid bodies, the first wave of caspase-independent cell death indispensable for mouse morphogenesis (Cande et al., 2002). AIF is neutralized by Hsp70, in a reaction that appears to be independent of ATP or the ABD of Hsp70 and thus differs from the Apaf-1/Hsp70 interaction (which requires ATP and the Hsp70 ABD) (Cande et al., 2002). Hsp70 lacking ABD (Hsp70ΔABD) inhibits apoptosis induced by serum withdrawal, staurosporin, and menadione, three models of apoptosis that are also affected by microinjection of anti-AIF antibody or genetic ablation of AIF (Cande et al., 2002; Ran et al., 2004). Altogether, these data suggest that AIF plays a role in the regulation of caspase-independent, mitochondrial-dependent cell death.

4.2 Hsp70 Regulation of Intrinsic Pathways of Apoptosis

Numerous proapoptotic molecules converge on mitochondria to produce mitochondrial outer membrane permeabilization (MOMP). These include proteins from the Bcl-2 family, mitochondrial lipids, proteins that regulate bioenergetic metabolite flux, and some components of the permeability transition pore (Green and Kroemer, 2004). MOMP results in cell death because of: release of molecules that activate caspase-dependent apoptosis; release of molecules that activate caspase-independent apoptosis; and loss of mitochondrial functions essential for survival of the cell. MOMP may occur via formation of a permeability transition (PT) pore on the inner membrane possibly via ANT or by actions of proapoptotic Bcl-2 protein family members on the outer membrane (Green and Kroemer, 2004). Proteins that act on the outer membrane include Bax, Bak, and Bid and may act alone or in concert with VDAC to produce MOMP. Once MOMP occurs, cytochrome *c*, AIF, Smac/Diablo, HtrA2/Omi, and endonuclease G are released from the matrix through the outer mitochondrial membrane that activate caspase-dependent and -independent apoptosis (Parcellier et al., 2003; Orrenius, 2004). Cytochrome *c* release is a two step process: first cytochrome *c* is released from cardiolipin, a phospholipid that anchors it to the outer surface of the

inner mitochondrial membrane; followed by release of cytochrome *c* through Bax/Bak forming pores in the outer membrane (Orrenius, 2004). Cytochrome *c* then interacts with Apaf-1 to begin formation of the apoptosome. Smac/Diablo and HtrA2/omi activate apoptosis by neutralizing the inhibitory activity of IAPs that inhibit caspases (Parcellier et al., 2003).

Hsp70 inhibits the intrinsic pathway upstream of the mitochondria and downstream of cytochrome *c* and upstream of caspase-3. Indeed, Hsp70 directly binds Apaf-1 thereby preventing recruitment of procaspase-9 to the apoptosome (Beere et al., 2000; Beere, 2001; Beere and Green, 2001). Hsp70 may also inhibit Apaf-1 oligomerization (Saleh et al., 2000). It is notable that Hsp90 is also reported to bind Apaf-1, making likely that the Hsp70/Hsp90/Hsp40 machine binds Apaf-1 and prevents formation of the apoptosome. The ATPase domain of Hsp70 is necessary for the interaction with Apaf-1 since deletion of the ATPase domain produces an Hsp70 molecule that does not prevent caspase activation (Beere et al., 2000; Mosser et al., 2000).

Hsp70 acts on pathways upstream of the mitochondria. Hsp70 binds to and functions as a natural inhibitory protein of c-jun N-terminal kinase (JNK1) (Jaattela et al., 1998; Park et al., 2001). JNK activation is potently suppressed by Hsp70 (Mosser et al., 2000Gabai and Sherman, 2002). This would prevent JNK phosphorylation of c-Myc, p53, Bcl-2, and Bcl-xl. JNK may also play a role in Smac release from the mictochondria (Chauhan et al., 2003). Hsp70 has also been reported to associate with p53, c-myc, and Raf-1/ERK, although the significance of these interactions for apoptosis is unclear. Hsp70 binding to p53 would prevent induction of PUMA that then acts on the mitochondria to produce MOMP.

4.3 Hsp70 Regulation of Extrinsic Pathways of Apoptosis

Hsps are reported to modulate signaling via all of the death receptors including Fas, TNF, and TRAIL. Although Fas-induced apoptosis usually proceeds via FADD and caspase-8, Fas can also signal through Daxx-ASK-1 to activate SAPK/JNK that precipitates mitochondrial-mediated apoptosis. The Fas-Daxx pathway is suppressed by Hsp70 binding to ASK-1 and JNK and by Hsp27 binding to Daxx (Beere, 2004).

The role of Hsp70 and other Hsps on TNF-induced cell death has been controversial, some reports having claimed that Hsp70 protected against TNF-mediated death (Jaattela, 1990; Jaattela and Wissing, 1993), whereas others indicated Hsp70 promoted TNF-induced cell death (Liossis et al., 1997; Schett et al., 2003). The role of Hsp70 in TNF-mediated cell death has recently been shown to relate to the balance between TNF-TNFR1 activation of the extrinsic apoptotic pathway (TRADD-RIP1, TRAF2–TRADD/DD-FADD–caspase-8) and RIP induction of NF-κB signaling and induction of antiapoptotic genes (Micheau and Tschopp, 2003). TNF added to Cos-1, Hela, and 293 cells does not kill them; however, marked overexpression of Hsp70 in the presence of TNF led to apoptosis (Ran et al., 2004). Hsp70 promoted TNF-mediated apoptosis by binding IKKγ and impairing NF-κB survival signaling and induction of antiapoptotic genes (Ran et al., 2004). Similarly, Hsp90 and Hsp27 also act on NF-κB signaling, Hsp90 binding IKKβ, and Hsp27 binding both IKKα and IKKβ to suppress NF-κB activity (Park et al., 2003). These binding activities might be predicted to decrease cell survival as found by Ran et al., which has been found in some but not in other studies (Mehlen et al., 1996; Pandey et al., 2000; Park et al., 2003). Indeed, Hsp90 also interacts with and stabilizes TIP-1 kinase, a protein that connects death receptors to NK-κB activation. In the absence of Hsp90, RIP-1 is degraded, which precludes NF-κB activation by TNFα and sensitizes the cells to apoptosis induced by TNF (Lewis et al., 2000). As for all of the Hsps, the net effect on cell survival or cell death by a given Hsp must be a complex interaction between multiple signaling pathways that may be different in different cells at different times.

The extrinsic and intrinsic pathways are coupled by caspase-8-mediated cleavage of Bid to tBid. tBid leads to Bax-dependent release of proapoptotic molecules from the mitochondria (Beere, 2004). Both Hsp70 and Hsp27 have been suggested to modulate Bid-induced apoptosis. As noted later, the Hsp27 effects on Bid-induced release of cytochrome *c* may be related to stabilization of actin microfilaments in the cytoskeleton rather than any direct effect of Hsp27 on mitochondria or cytochrome *c* (Beere, 2004). Hsp70 has been reported to prevent cleavage and activation of Bid in response to TNF—an effect independent of

its chaperoning function (Gabai et al., 2002). This is somewhat surprising in view of the findings of Ran et al. (2004) that Hsp70 can facilitate TNF-mediated cell death as noted earlier. This effect might not be direct, however, since Hsp70 also suppresses activation of the MAP kinase JNK pathway that can stimulate cytochrome *c* and Smac release from mitochondria (Beere, 2004).

Hsp70 can also facilitate caspase activation in granzyme B-mediated apoptosis and Hsp70 enhances cytototoxic T lymphocyte killing (Beere, 2004). Hsp70 and Hsc70 can enhance T-cell receptor-mediated apoptosis by directly associating with CAD to augment its activity.

4.4 Other Heat-Shock Proteins Regulate Apoptosis: Hsp27, Hsp60, Hsp10

Hsp27 belongs to the family of small Hsps of which there are at least nine family members (Concannon et al., 2003). Hsp27 is a potent regulator of actin microfilaments. Hsp27 is expressed at low levels in the brain and is induced mainly in astrocytes in the brain. Small Hsps share a C-terminal domain called the crystalline box that comprises 100 amino acids in the C terminus of the protein and has an IgG-like fold (Concannon et al., 2003). The p38Map kinase phosphorylates MAPKAP kinases 2/3 to phosphorylate Hsp27 on three different serine residues. Hsp27 functions as a chaperone to aid in the refolding of nonnative proteins, but unlike Hsp70, Hsp27 helps refold proteins in an ATP-independent manner. Hsp27 enhances the rate of recovery from nuclear protein aggregation (Concannon et al., 2003). Hsp27 also regulates various components of apoptotic pathways.

Hsp27 is reported to directly bind cytochrome *c* and impair formation of the apoptosome (Garrido et al., 2001). The heme group of cytochrome *c* is necessary but not sufficient for the interaction with Hsp27 (Parcellier et al., 2003). However, the observations related to direct binding have been disputed (Concannon et al., 2003). Instead, Hsp27 may (also) stabilize the actin cytoskeleton and prevent translocation of proapoptotic factors from the actin cytoskeleton to mitochondria where they cause cytochrome *c* release (Paul et al., 2002). Hsp27 has also been reported to decrease apoptosis by blocking Smac release in myeloma cells where it did not block cytochrome *c* release (Beere, 2004). Hsp27 is also reported to affect the death receptor-mediated apoptotic pathway. This appears to be due to Hsp27 binding to Daxx—notable because only phosphorylated Hsp27 appears to bind Daxx (Paul et al., 2002; Concannon et al., 2003). Both Hsp27 and α-crystalline interact with procaspase-3 and might explain findings that Hsp27 can modulate death-receptor-mediated cell death (Beere, 2004).

Hsp60 and Hsp10 function within mitochondria to fold proteins imported into mitochondria, to fold proteins synthesized within mitochondria, and to refold misfolded proteins. In doing so, they protect the mitochondria and the cell (Garrido et al., 2001). However, it is also reported that Hsp60 and Hsp10 can be released from mitochondria, perhaps in late stages of apoptosis or with certain types of mitochondrial damage, after which they associate with procaspase-3 and favor its activation (Samali et al., 1999).

Hsp90 is also reported to modulate apoptosis. Hsp90 may also directly bind Apaf-1 and block the formation of the apoptosome and the execution of apoptosis via the intrinsic pathway (Garrido et al., 2001). Hsp90 also stabilizes RIP-1 which is critical for NF-κB activation and activation of NF-κB induction of antiapoptotic genes (Garrido et al., 2001). Hsp90 also binds Akt to prevent its dephosphorylation and deactivation (Garrido et al., 2001).

5 Heat-Shock Proteins: Disease and Neuroprotection

5.1 Role of Heat-Shock Proteins in Neurological Disease: Acute Injury

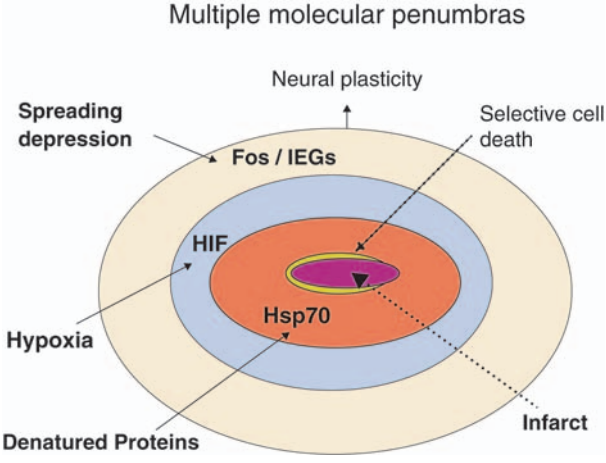
Virtually all of the heat genes are induced in the brain following focal and global ischemia/stroke and other types of acute injury that damage or kill cells including status epilepticus, hypoglycemia, and trauma (Tang et al., 2002). Because Hsp70 protein is induced in response to denatured proteins in cells, Hsp70 expression has been used as an indicator of protein stress in cells following cerebral ischemia. In addition, it has been

proposed that Hsp70 protein expression denotes those stressed cells in the penumbra that are injured but will likely survive the ischemia. The zone of Hsp70 protein expression outside the infarct could represent the "classical penumbra" (Kinouchi et al., 1993a, b; Hata et al., 2000; Sharp et al., 2000).

Following stroke, Hsp70 mRNA is induced in all cell types in the center of the infarction and is induced mainly in isolated neurons outside the zone of infarction (Kinouchi et al., 1993a, b). Hsp70 protein expression, however, is quite different. Within the core of the infarction either no Hsp70 protein is expressed, or Hsp70 protein is expressed only in blood vessels. It was suggested that expression in the blood vessels meant that these cells would survive (Kinouchi et al., 1993a, b). At the margins of the infarcts Hsp70 protein was expressed in astrocytes and microglia and in neurons. Outside the areas of infarction there was a thin rim of TUNEL positive, Hsp70 unstained neurons. Of note Hsp70 protein is not expressed in TUNEL stained, apoptotic neurons, suggesting cells that make the choice of dying via apoptosis do not also express Hsp70 protein and vice versa (States et al., 1996). In addition, Hsp70 protein was expressed in neurons at some distance from the infarction. The zone of Hsp70 stained neurons was large with short durations of focal ischemia and was quite small with very prolonged durations of ischemia (Kinouchi et al., 1993a, b). The Hsp70 mRNA and Hsp70 protein expression in neurons outside the infarction occurs in the zone where protein synthesis is suppressed, but ATP is maintained (Hata et al., 2000). Thus, Hsp70 protein expression in neurons outside the area of infarction is proposed to occur in the "penumbra" that can be rescued by pharmacological treatments (Kinouchi et al., 1993a, b; Sharp et al., 2000) (*Figure 6-5*).

Figure 6-5

Multiple molecular penumbras surround an area of focal cerebral infarction. The infarct is surrounded by a thin zone of dead cells, which are mainly apoptotic neurons. Hsp70 protein is expressed in neurons outside of the infarction in a region of suppressed protein synthesis but maintained ATP, a region that probably fulfills the definition of the "classic penumbra." Adapted from Sharp et al. (2000)

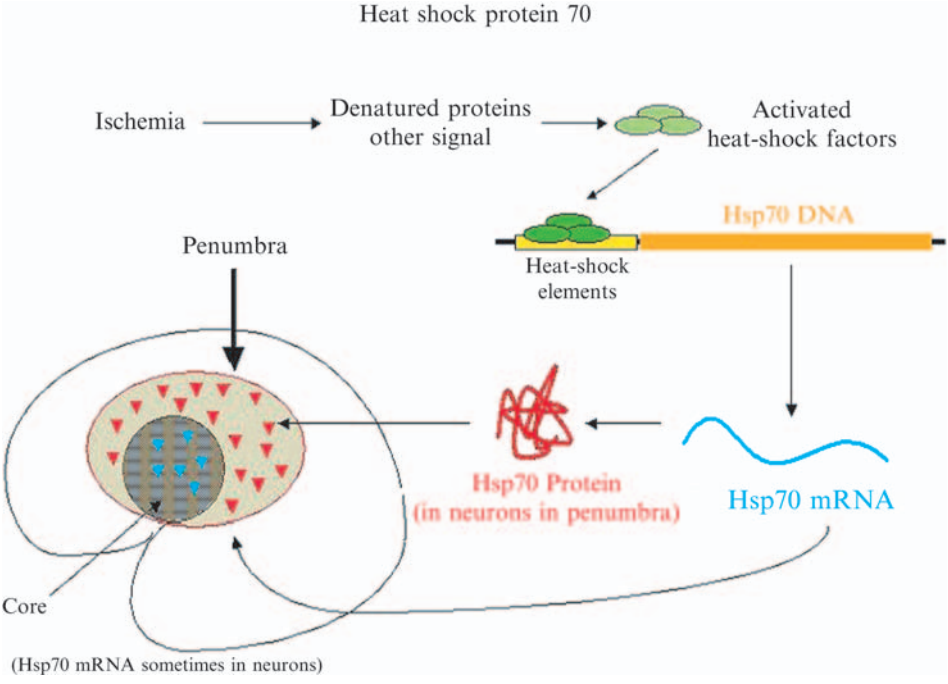


Other types of acute injury also induce Hsp70 as well as other Hsps. Subarachnoid injections of the blood produce multifocal areas of Hsp induction where Hsp70, Hsp32, and Hsp40 are induced. It was proposed that these represent areas of vasospasm associated with focal ischemia and Hsp induction without any evidence of focal infarction (Turner et al., 1999). In addition, subarachnoid injections of the blood or purified hemoglobin-induced HO-1 in microglia throughout the entire brain. It was proposed that heme in hemoglobin is specifically transported into microglia throughout the brain and that this induces Hsp32/ also called HO-1. HO-1 could serve to chaperone the heme in the brain as well as metabolize heme to

biliverdin that is then metabolized to bilirubin and removed from the brain (Matz et al., 1996; Turner et al., 1998) (*Figure 6-6*).

Figure 6-6

Hsp70 is induced in the brain following focal cerebral ischemia by denatured proteins and other signals within damaged cells. HSFs are activated/phosphorylated by the appearance of the denatured proteins within the cells, and HSF trimers then bind to HSEs in the Hsp70 genes, and in other heat-shock genes, to activate Hsp70 transcription. Hsp70 mRNA is then synthesized in neurons inside the core of the focal infarction as well as in neurons outside the infarct. Hsp70 protein, however, is not synthesized in the neurons in the core of the infarct. Hsp70 protein is synthesized in the neurons in the penumbra around the infarct and serves to help these neurons survive if ATP levels are maintained in these cells. Adapted from Sharp et al. (2000)



5.2 Heat-Shock Proteins Role in Neurodegenerative Diseases

A number of neurodegenerative diseases involve protein misfolding and aggregation that often results in cellular inclusions and stimulation of the Hsp response. Although it is not possible to review all of these diseases, the basic ideas involved will be summarized.

Huntington's disease is caused by expansion of a CAG repeat coding for polyglutamine in the N terminus of the huntingtin protein. There is a striking correlation between the threshold for aggregation in vitro and the threshold for disease in humans. Other polyglutamine diseases including dentate-rubral-pallido-luysian atrophy and several forms for spinocerebellar ataxia also have in intranuclear inclusions. However, the inclusions in Huntingtons and in the SCAa do not always correlate with cell death. In Alzheimer's disease extracellular aggregates known as neuritic plaques contain Aβ peptide, which is derived from proteolytic processing of the amyloid precursor protein. There are also intracellular aggregates of the

microtubule-associated protein tau, called neurofibrillary tangles. Familial forms of Alzheimer's disease are caused by mutations in APP and in presenilins that are involved in APP cleavage. Parkinson's disease is associated with cytoplasmic inclusions called Lewy bodies that are aggregates of α-synuclein protein. Familial Parkinson's disease can be caused by mutations in α-synuclein. Familial amyotrophic lateral sclerosis (ALS) due to mutations in the SOD1 gene is associated with cytoplasmic inclusions containing SOD1 protein aggregates (Sherman and Goldberg, 2001; Ross and Poirier, 2004). There is substantial evidence that protein damage, misfolded proteins, and/or abnormal protein aggregates can induce apoptosis in neuronal and other types of cells. One example of this is where amino acid analogs are given to cells that result in formation of improperly folded proteins that cause apoptosis without formation of cellular inclusions. The formation of malformed proteins would be expected to stimulate a stress/heat-shock response that may or may not be effective in either refolding the proteins or in properly processing them for degradation either in the proteasome, by proteases or by lysosomes (Sherman and Goldberg, 2001). Presumably, if the heat-shock response does not occur, or the response is not sufficient, or if the heat-shock response cannot properly rid cells of the malformed proteins, this could lead to apoptosis.

Hsc70 and Hsp40 associate with mutant hungtintin and ataxin-3 in aggregesomes in striatal neurons. Proteasome inhibitors increased inclusion bodies and apoptosis (Sherman and Goldberg, 2001). Expression of ataxin-1 or ataxin-3 and Huntingtin with expanded polyglutamines in cultured cells induces Hsp70.

Neuronal protection has been achieved in a number of disease models by the overexpression of Hsp70 and other chaperones. Overexpression of Hsp70 and Hsp40 protects Drosophila neurons with forced expression of mutant forms of human Huntington (Bonini, 2002). Hsp70 and Hsp40 protect against apoptosis induced by the mutant androgen receptor that causes spinal bulbar muscular atrophy (Sherman and Goldberg, 2001). Overproduction of Hsp104 in *C. elegans* reduces neurotoxicity of mutant huntingtin. Hsps reduce the formation of inclusion bodies by huntingtin fragment with an expanded polyglutamine and decrease aggregation. Yeast Hsp104 is essential for degradation of ataxin-1. Overexpression of inducible Hsp70 or Hsp40 chaperone suppresses protein aggregation in cells and improves neuropathology and motor function in SCA1 mice (ataxin-1 mutant mice) (Cummings et al., 1998, 2001). Hsp70 together with Hsp40 markedly protects Drosophila dopamine neurons against enforced α-synuclein overexpression in a fly model of Parkinson's disease (Bonini, 2002). Prior expression of the chaperones also prevents the disease in the flies. Of note in the fly studies is that overexpression of Hsp70 and Hsp40 do not produce any change in the cellular aggregates or inclusions but appear to improve the solubility of the proteins in the aggregates or perhaps prevent interactions with other proteins in the cell that might make them toxic (Bonini, 2002).

Hsc70 is also involved in protein degradation, in a process called chaperone-mediated autophagy (CMA) (Cuervo et al., 2004). For example, Hsc70 binds normal α-synuclein and targets it to a lysosomal membrane receptor, lamp2a. The α-synuclein is then translocated into the lysomal lumen where it is degraded by hydrolases. Mutant α-synucleins that accumulate in Lewy inclusion bodies and result in Parkinson's disease in humans, appear to act as uptake blockers, inhibiting both their own degradation and that of other Hsc70 substrates (Cuervo et al., 2004). These observations account for the inclusions seen in familial Parkinson's disease and may help explain the mechanism of cell death and the toxic gain-of-function by the mutants (Cuervo et al., 2004).

Mutations of Parkin cause a juvenile form of Parkinson's disease. Death of dopaminergic neurons occurs because of accumulation of unfolded Pael receptor in the endoplasmic reticulum without formation of aggregates. Parkin is an E3 ubiquitin ligase that forms a complex with Pael-R, CHIP, and Hsp70. In such a complex, CHIP facilitates Hsp70 dissociation from Park and Parkin-mediated ubiquitination of Pael-R. Mutations of Parkin thus result in Pael-R accumulation in an unfolded state (Garrido and Solary, 2003).

A novel mechanism of disease was recently described in which RNA-mediated neurodegeneration was caused by the fragile X permutation rCGG repeats in Drosophila (Jin et al., 2003). The mechanism by which this occurred was speculated to be that the CGG mutation-repeat induced misfolding of one or more RNA-associated proteins. This would trigger the cellular misfolded protein response, resulting in toxic protein aggregates that eventually kill cells (Jin et al., 2003).

5.3 Heat-Shock Proteins also Protect Against Acute Brain Injury

One of the early and most important observations related to the heat-shock response was that moderate heat exposure of flies and cells to levels to induce Hsps, but which did not kill cells or the organism, led to protection against subsequent lethal heat-shock delivered within a few days (Lindquist, 1986, 1992). This protection was related to expression of Hsp70 (Lindquist, 1992), and in yeast to expression of Hsp100 family members (Lindquist and Kim, 1996).

These findings have been translated to acute injury to the brain and other organs (Li et al., 1992; Mosser et al., 1997; Trost et al., 1998; Latchman, 2001). Barbe et al. (1988) first showed that heating protected the mammalian retina against light-induced injury, and this protection correlated with the induction of Hsp70. Overexpression of Hsp70 in cells in vitro protects them against a variety of injuries including ischemia (Yenari, 2002). Viral overexpression of Hsp70 and transgenic overexpression of Hsp70 in vivo also protect the brain against ischemia and other types of injury (Sharp, 1998; Yenari et al., 1999; Yenari, 2002; Rajdev et al., 2000). Indeed, estrogen induction of Hsps in cerebral vessels also protects the brain against ischemia (Lu et al., 2002b), and inducing Hsps with Hsp90-binding drugs also protects the brain against cerebral ischemia (Lu et al., 2002a).

6 Conclusions

Hsps play critical roles in all cellular processes including protein synthesis, protein folding, protein refolding, protein transport, protein degradation, and ultimately in cell survival and cell apoptosis. It is not surprising, therefore, that Hsps are implicated in the regulation of many cell-signaling pathways and in protecting against or perhaps even mediating many neurological diseases. They are sure to continue to be on center stage in neurological diseases including those related to neural development.

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7 Cotranslational Protein Folding and Aggregation After Brain Ischemia

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Abstract: Folding of newly synthesized polypeptide occurs at the level of the protein domain; folding does not take place until a whole protein domain has been synthesized on the ribosome. This folding process during polypeptide elongation on a ribosome is referred to as cotranslational folding. Cotranslational folding is a sophisticated cellular process that often requires a cooperation of chaperones, cochaperones, and cellular ATP. Brain ischemia leads to protein aggregation in neurons, and thus it is a proteotoxic stress. Emerging evidence indicates that protein aggregation takes place on ribosomes, thus damaging protein synthesis machinery in neurons and contributing to the pathogenesis of brain ischemia. At least two cellular systems are involved in cellular defense of proteotoxicity after brain ischemia: molecular chaperones and the ubiquitin–proteasomal system. This chapter discusses recent advances regarding the aggregation of cotranslational folding complexes after brain ischemia.

List of Abbreviations: 40S, small ribosomal subunit; 60S, large ribosomal subunit; CA, cornu ammonis; DG, dentate gyrus; eIF, eukaryotic initiation factor; EM, electron microscopy; EPTA, ethanolic phosphotungustic acid; ER, endoplasmic reticulum; HSC, heat shock cognate protein; HSP, heat shock protein; L28, large ribosomal subunit protein 28; S6, small ribosomal subunit protein 6; ubi-proteins, ubiquitin-conjugated proteins; UPR, unfolded protein response

1 Introduction

It was found in the 1970s that neuronal death does not occur immediately but takes place over several days following an episode of cerebral ischemia. Such a slowly progressing type of neuronal death is referred to as the maturation phenomenon, or delayed neuronal death (Kirino, 1982; Ito et al., 1975). In forebrain rat ischemia models, transient cerebral ischemia followed by reperfusion causes neuronal death selectively in hippocampal CA1 pyramidal neurons after 48–72 h of reperfusion while leaving dentate gyrus (DG) granule cells largely intact. During the 48–72 h delay period, CA1 neurons destined to die appear essentially normal under the light microscope (Smith et al., 1984). At the electron microscopic level, however, disaggregation of ribosomes, deposition of dark substances or materials, abnormalities in the Golgi apparatus, and damage of synapses have been reported (Kirino et al., 1984; Petito and Lapinski, 1986; Deshpande et al., 1992; Tomimoto and Yanagihara, 1992; Rafols et al., 1995; Hu et al., 1998; Martone et al., 1999). Delayed neuronal death after an initial insult also occurs after other brain injuries such as in the penumbra region after focal ischemia and in some brain regions after hypoglycemia (Auer et al., 1985; Nedergaard, 1987; Ouyang and Hu, 2001). In this chapter, I will review evidence that ischemia demolishes protein synthesis machinery by aggregation of cotranslational folding complexes, thereby contributing to delayed neuronal death after brain ischemia.

2 Protein Aggregation After Brain Ischemia

The understanding of protein aggregation and proteotoxicity in pathological conditions has become one of the greatest challenges facing modern medicine because proteotoxicity is related to a broad range of conformational diseases. Neurons are particularly vulnerable to the toxic effects of protein unfolding and misfolding, which is underscored by the fact that protein aggregation occurs in virtually all neurodegenerative disorders. Recently, aberrant protein homeostasis or proteotoxicity has been implicated in playing a critical role in delayed neuronal death after brain ischemia (Hu et al., 2000, 2001; Ouyang and Hu, 2001; Hu et al., 2004). The importance of proteotoxicity in ischemic delayed neuronal death is further accentuated by studies showing that introducing molecular chaperones by transgenic gene expression or viral vector gene delivery protects neurons from ischemia (Sharp et al., 1999; Yenari et al., 2001; Giffard et al., 2004). In addition, the acquisition of stress tolerance or preconditioning has been widely considered to be brought about by means of activation of molecular chaperones and the ubiquitin–proteasomal systems (Kirino, 2002).

Many hypotheses regarding molecular mechanisms underlying delayed neuronal death after brain ischemia have been proposed (Siesjö and Siesjö, 1996). Among them, persistent inhibition of protein

synthesis (Cooper et al., 1977; Hossmann, 1993), overproduction of reactive oxygen species (Chan, 1996), impairment of protein ubiquitination and increases in ubiquitinated proteins (ubi-proteins) (Magnusson and Wieloch, 1989; Hayashi et al., 1993; Morimoto et al., 1996), and expression of stress proteins (Nowak, 1985; Rafols et al., 1995; Sharp et al., 1999; Yenari et al., 2001) have been postulated to be important for delayed neuronal death or survival after ischemia. In early studies, dark substances or materials were observed by electron microscopy in CA1 neurons after transient ischemia, although the identities of these dark materials were unknown (Kirino et al., 1984; Deshpande et al., 1992; Tomimoto and Yanagihara, 1992). Are these events independent or are they associated with a common underlying event? Recent studies support a notion that these intracellular events may be commonly related to overproduction of abnormal proteins and their aggregation after brain ischemia (Hu et al., 2000, 2001, 2004; Ouyang and Hu, 2001; Liu and Hu, 2004). In an early ethanolic phosphotungstic acid (EPTA) electron microscopic study, large quantities of abnormal aggregates stained with EPTA were observed in the somas and dendrites of CA1 neurons after ischemia (*Figure 7-1a*). The EPTA staining method has frequently been used as an electron microscopic selective staining method for synaptic structures and nuclei (Bloom and Aghajanian, 1966). Because EPTA preferentially reacts with proteins (Burry and Lasher, 1978), the aggregates stained with EPTA are likely composed of proteins. This conclusion is supported by the fact that these intraneuronal aggregates contain strong ubiquitin immunoreactivity as demonstrated by immunogold electron microscopy and confocal microscopy (Hu et al., 2000, 2001, 2004). Therefore, proteins may be severely unfolded, abnormally modified, or damaged, and eventually aggregated in ischemic vulnerable neurons (Hu et al., 2000, 2001).

Consistent with the data obtained from electron microscopic studies, transient cerebral ischemia induces a massive aggregation of ubi-proteins during the postischemic phase. Ubi-protein redistribution can be studied using high-resolution confocal microscopy (Hu et al., 2000, 2001). The patterns of ubiquitin and ubi-protein immunostaining in CA1 neurons following an episode of brain ischemia is altered continuously from a relatively even distribution in sham-operated controls to large and patchy distribution surrounding the nuclei and attached to the dendritic membranes during reperfusion (*Figure 7-1b*). This pattern at 24 h of reperfusion is virtually similar to the distribution of protein aggregates stained with EPTA under electron microscopy (*Figure 7-1a*).

Results of morphological and immunocytochemical studies were also supported by biochemical evidence. Using western blotting, it was found that ubi-proteins were dramatically increased as early as 30 min of reperfusion and persisted until cell death in CA1 neurons (*Figure 7-2a*). However, ubi-proteins can be extracted by 2% Triton X100 from brain tissues at 30 min of reperfusion (*Figure 7-2b*), suggesting that many ubi-proteins have not yet aggregated at 30 min of reperfusion because protein aggregates are detergent insoluble (*Figure 7-2b*). Most ubi-proteins gradually become detergent insoluble after 4–24 h of reperfusion, suggesting that these ubi-proteins are irreversibly aggregated in CA1 neurons after ischemia (*Figure 7-2b*) (Hu et al., 2000; Liu and Hu, 2004). These results are in line with several previous studies demonstrating depletion of intracellular free ubiquitin and increase in ubi-proteins after ischemia (Magnusson and Wieloch, 1989; Hayashi et al., 1993; Morimoto et al., 1996). Evidence suggests that depletion of free ubiquitin immunostaining reflects an overproduction of abnormal proteins that are first ubiquitinated, from which some ubi-proteins are irreversibly aggregated in ischemic vulnerable neurons (Hu et al., 2000, 2001).

3 Aggregation of Cotranslational Folding Complexes After Brain Ischemia

Newly synthesized proteins are the major sources of unfolded proteins in normal cells (Bukau et al., 1996; Frydman, 2001; Kusmierczyk and Martin, 2001; Hartl and Hayer-Hartl, 2002). Are newly synthesized polypeptides aggregated after ischemia? The ribosome is the protein synthesis machine and consists of a 40S small subunit and an 80S large subunit in mammalian cells. Both subunits are composed of RNAs and proteins. The small subunit mediates the interactions among protein synthesis initiation factors, transfer RNAs (tRNAs), and messenger RNAs (mRNAs) to determine the sequences of the proteins being made. The large subunit catalyzes peptide bond formation for peptide elongation. The newly synthesized peptide on the ribosome is designated as the nascent peptide chain. During translation, the C-terminal end of a nascent

Figure 7-1

Brain sections were from sham-operated control rats and rats subjected to 15 min of cerebral ischemia followed by 24 h of reperfusion. (a) Electron micrographs of EPTA-stained CA1 pyramidal neurons. EPTA strongly stains synaptic structures (*arrows*) and nucleus (N) but relatively weakly reacts with other subcellular structures in a sham-control CA1 neuron. EPTA not only stains synaptic structures (*small arrows*) and nucleus (N) but also additionally labels abnormal protein aggregates in CA1 neurons after ischemia. Abnormal protein aggregates distribute within perikarya of CA1 pyramidal neurons and associate with dendritic plasmalemma (*large arrows*). (b) High magnification laser-scanning confocal microscopic images of CA1 pyramidal neurons double labeled with an anti-ubi-protein antibody (green) and propidium iodide (red). Magnification was increased using the zoom function in the confocal software. Ubiquitin immunoreactivity (green color) is relatively evenly distributed in sham-control CA1 neuronal somata and dendrites (*arrows*), but appears as patchy aggregates surrounding the nuclei and attached to the dendritic plasmalemma (*arrows*), and disappears from nuclei at 24 h of reperfusion after ischemia. Propidium iodide-stained CA1 nuclei (red) appear unchanged after ischemia

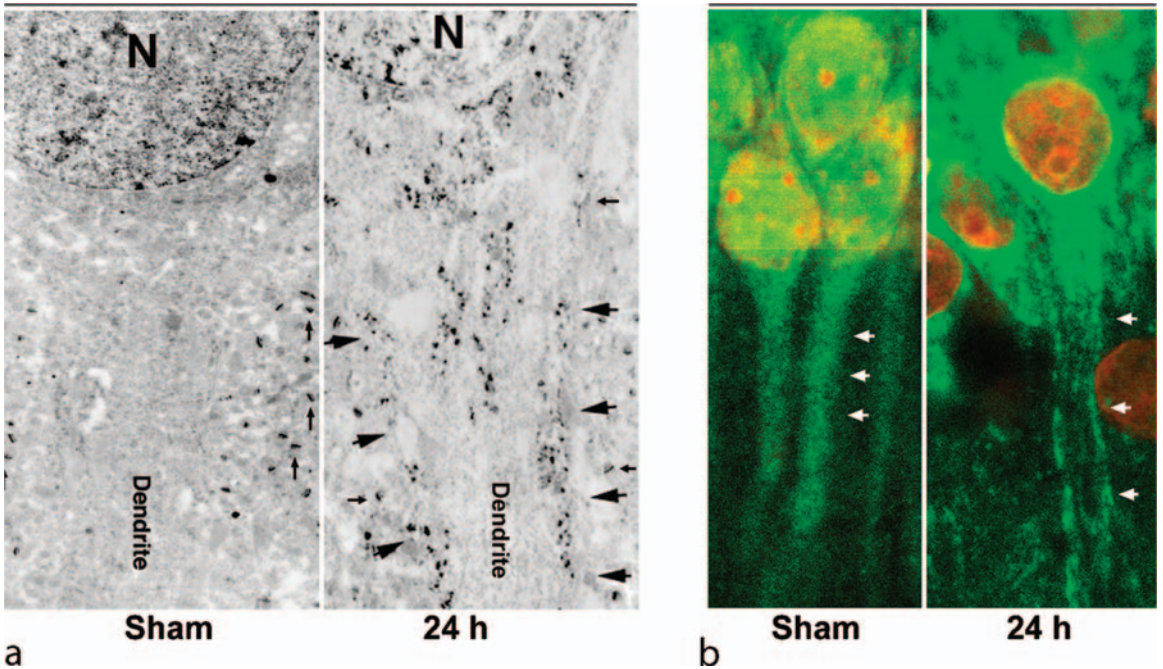
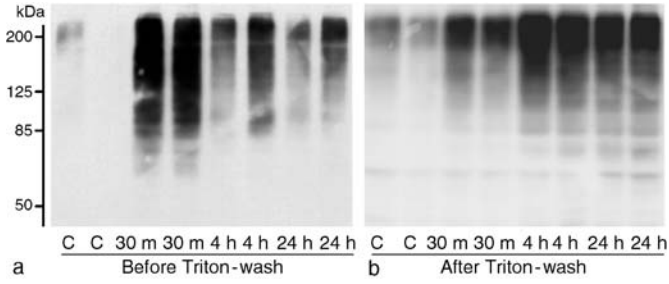


Figure 7-2

Immunoblots of ubi-proteins in the pellet fractions (a) before and (b) after 2% Triton X100 wash. The CA1 tissues were dissected from (c) sham-operated control rats and rats subjected to 15 min of cerebral ischemia followed by 30 min, 4h, and 24 h of reperfusion. Two separate samples in each experimental group were run in parallel on SDS-PAGE. The blots were labeled with an anti-ubi-protein antibody and visualized with the ECL system. Molecular size markers are indicated on the left



peptide is bound as a peptidyl-tRNA to the large ribosomal peptidyl transferase center, whereas the N terminus of the nascent peptide emerges from the large ribosome (Hardesty et al., 1999; Rospert, 2004).

Translating genetic information encoded in DNA into a linear polypeptide on a ribosome is normally done with fidelity (Frydman, 2001). However, to become a nontoxic functional protein, a newly synthesized polypeptide has to overcome a key rate-limiting hurdle—folding into a unique three-dimensional conformation (Bukau et al., 1996; Frydman, 2001; Kusmierczyk and Martin, 2001; Hartl and Hayer-Hartl, 2002). Folding of newly synthesized polypeptide occurs at the level of the protein domain or multidomains; folding may not take place until a whole protein domain has been synthesized on the ribosome. This folding process during polypeptide elongation on a ribosome is referred to as cotranslational folding (Hardesty et al., 1999; Frydman, 2001; Hartl and Hayer-Hartl, 2002). Because ribosomes are highly abundant in cells, many nascent polypeptides are often emerging from ribosomes at any given moment. Newly synthesized polypeptide chains expose their hydrophobic segments and are highly prone to intramolecular misfolding and intermolecular aggregation driven by the hydrophobic force (Hartl and Hayer-Hartl, 2002). Therefore, cotranslational folding generally requires: (1) assistant or protective chaperone proteins; (2) their cochaperones; and (3) cellular energy supply. If a newly synthesized protein cannot reach its final conformation, it is normally degraded immediately by the ubiquitin–proteasomal system to avoid aggregation. Cotranslational folding is a very complicated process and must cooperate with protein folding and the degradation machinery in the crowded cellular milieu (Frydman, 2001). Any errors either due to wrong genetic coding or due to dysfunction of protein folding machinery may cause human disease. It is, therefore, not a surprise to see that numerous human diseases, particularly neurological diseases, are caused by protein misfolding and aggregation.

The assistant or protective proteins for protein folding are generally referred to as molecular chaperones. In general, chaperones are able to recognize and shield exposed hydrophobic segments. These hydrophobic segments should normally be buried inside folded cytosolic proteins or assembled into lipid membranes. There are several families of chaperones and cochaperones in the cytosol involved in regulation of protein folding, trafficking, and degradation. The best known chaperones for cotranslational folding are the heat-shock protein 70 (HSP70) family and heat-shock protein 40 (HSP40) family. As their names imply, these chaperone proteins can be induced under hyperthermic or other stresses to facilitate cotranslational folding (Li et al., 1995). The HSP70 family in mammalian cytosol consists of a constitutive HSP70 cognate form of HSC70 and a stress-inducible form of HSP70. HSC70 is the major form involved in cotranslational folding in normal cells. HSC70 contains an N terminus ATPase domain and a C terminus peptide-binding domain and carries out cycles of binding and release of nascent polypeptides during cotranslational folding in an ATP-dependent manner. To compete with aggregation, complex formation between HSC70 and its nascent peptide substrate must be more efficient than nascent chain aggregation. The kinetics of the substrate binding and release cycles coupled by HSC70 alone is too slow to handle the large quantities of

nascent chains and to compete with nascent chain aggregation in normal cells and thus must be accelerated by a cochaperone HSP40 that promotes HSC70 hydrolysis of ATP (Frydman et al., 2001). HSP40 contains various J-domains through its structure that stimulate HSC70 ATPase activity (Ohtsuka and Hata, 2000). It is proposed that HSP40 first binds to a nascent chain. The HSP40–substrate complex then associates with HSC70 and ATP to transfer the nascent chain to HSC70 by hydrolysis of ATP. This cooperative interaction among HSP40, HSC70, and ATP hydrolysis shifts HSC70 to its low-affinity mode and allows release of bound polypeptide from HSC70 (Hartl and Hayer-Hartl, 2002). Cotranslational folding of nascent peptide usually requires many cycles of HSC70-mediated ATP binding and hydrolysis. During this process, HSC70 must release ADP for rebinding to ATP in order to start another cycle, which is facilitated by a nucleotide exchange factor. Bcl-2-associated athanogene (BAG-1) functions as a nucleotide exchange factor for mammalian cytosolic HSC70, thereby triggering substrate unloading from the HSC70 chaperone (Alberti et al., 2003). Therefore, HSC70, HSP40, BAG-1, and ATP must cooperatively regulate the binding-release cycles in order to avoid newly synthesized polypeptide chain misfolding and aggregation. In addition, some proteins require further posttranslational folding, which is assisted by the HSP60 and HSP90 families of chaperones (Hartl and Hayer-Hartl, 2002).

If nascent chains cannot be folded successfully as a result of translational errors, the complex of a nascent polypeptide and its associated chaperones must recruit ubiquitin ligases like HSC70-interacting protein (CHIP) for degradation. CHIP is a cytoplasmic protein highly expressed in the brain and muscles and contains an N terminus tetratricopeptide repeat (TPR) domain and a C terminus U-box domain that has E3 ubiquitin ligase activity (Murata et al., 2003). CHIP binds to the HSC70–substrate complex through its TPR domain and conjugates HSC70 substrate with ubiquitin by its ubiquitin ligase activity. This interaction results in ubiquitination of chaperone-bound substrate and degradation of the substrate by proteasomes, thus, linking the wrongly folded peptide to the proteasomal pathway. During this process, BAG-1 may be also recruited to the complex and acts as a nucleotide exchange factor to facilitate the release of chaperones from the degradation complex. Therefore, CHIP and BAG-1 cooperate to direct an unsuccessfully folded nascent chain to the ubiquitin–proteasomal pathway.

Brain ischemia may disable cotranslational folding and degradation machinery, resulting in abnormal aggregation of cotranslational folding complexes together with their associated ribosomes after ischemia. When cellular ATP is decreased to below 80% of its normal cellular level, all ATP-dependent cellular processes are stopped or markedly slowed down (Siesjo and Siesjo, 1996). ATP-dependent cellular processes include maintenance of cellular ionic homeostasis (Siesjo and Siesjo, 1996), cotranslational folding (Hardesty et al., 1999), protein chaperoning (Hartl and Hayer-Hartl, 2002), and ubiquitin–proteasome-mediated protein degradation (Alves-Rodrigues, 1998). The common result of these ischemia-induced cellular alterations can lead to the overload of unprocessed nascent polypeptides on ribosomes after ischemia. The following evidence suggests that overload of unfolded nascent polypeptides on ribosomes may lead to their abnormal aggregation and irreversibly damage protein synthesis machinery after brain ischemia: (1) electron microscopy shows that ribosomes are abnormally aggregated in CA1 neurons after ischemia; (2) ribosomal proteins and cotranslational chaperone–cochaperone complexes are highly accumulated into the detergent-insoluble protein aggregate-containing fraction during the postischemic phase; (3) despite the fact that depletion of ATP is virtually recovered in neurons during reperfusion, the overall rate of protein synthesis is irreversibly inhibited in ischemic vulnerable neurons; (4) persistent inhibition of protein synthesis is a hallmark of delayed neuronal death, i.e., neurons with persistent inhibition of protein synthesis eventually die in a delayed fashion (Cooper et al., 1977; Hossmann, 1993; Hu et al., 1993). Persistent protein synthesis inhibition after ischemia could not be explained by a brief (about 30 min) eIF-2α phosphorylation (Burda et al., 1994; DeGracia et al., 1996, 2002). Emerging evidence strongly suggests that irreversible inhibition of protein synthesis in vulnerable neurons is caused by protein aggregation on ribosomes after ischemia.

Two major types of protein aggregates can be found in ischemic vulnerable neurons: ribosomal structure-like and vesicle-membrane-associated aggregates. This chapter focuses on the discussion of cotranslational ribosomal folding complex aggregation. The vesicle-membrane-associated protein aggregation after ischemia has been discussed elsewhere (Hu et al., 2004; Liu and Hu, 2004; Liu et al., 2004). Both conventional and EPTA electron microscopic studies demonstrate that ribosomal structure-like aggregates

are a major form of protein aggregates accumulated in ischemic vulnerable neurons. Ribosomal structures can be seen in both conventional osmium–uranium–lead sections and EPTA-stained sections by electron microscopy, although the EPTA-staining method relatively weakly stains ribosomes (Hu et al., 2000). However, ribosomes in EPTA-stained sections can be counterstained with the ribosomal staining method (*Figure 7-4*, *lower panel*). Under electron microscopy, most ribosomes are stained as rosettes and orderly distributed in cytosol and appear as ribosomal studs attached to the endoplasmic reticulum (ER) in sham-operated control neurons (*Figure 7-3*). After ischemia, most ribosomal rosettes disaggregated into single

Figure 7-3

Electron micrographs of osmium–uranium–lead–stained CA1 pyramidal neurons from a sham-operated control rat and rats subjected to 15 min of cerebral ischemia followed by 4 and 24 h of reperfusion. *Upper panel*: the rough endoplasmic reticulum (ER), mitochondria (M), nucleus (N), Golgi apparatus (G), and ribosomal rosettes (*arrows*) are normally distributed in sham-operated CA1 neurons. At 4 and 24 h of reperfusion after ischemia, the ER and mitochondria (M) are dilated, membranous vesicles (V) are accumulated, and ribosomes (*arrow*) are clumped into large aggregates. *Lower panel*: higher magnification of the ribosomal area indicated in the *upper panel* with three black arrows in sham, 4, and 24 h micrographs. Ribosomal rosettes and ER-associated ribosomes are normally distributed in sham-operated control CA1 neurons (*arrows*). After ischemia, ribosomes are abnormally clumped into large aggregates (arrows). Scale bars: 1 μm in the *upper panel* and 0.25 μm in *lower panel*

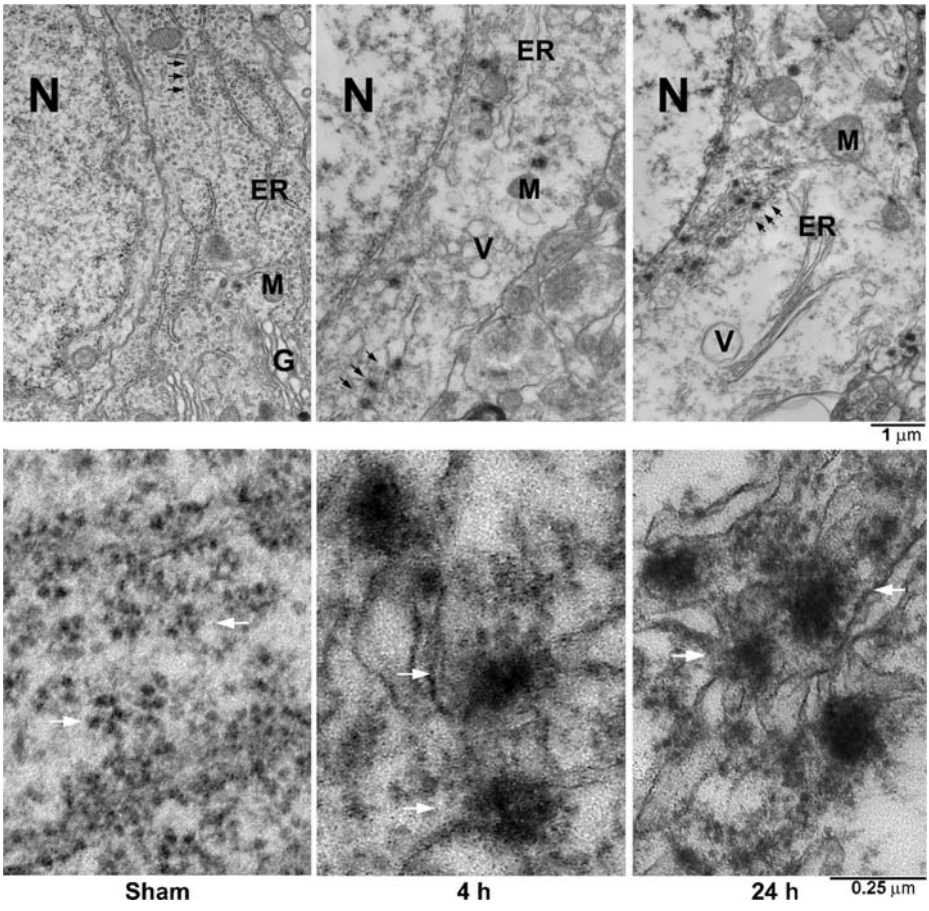
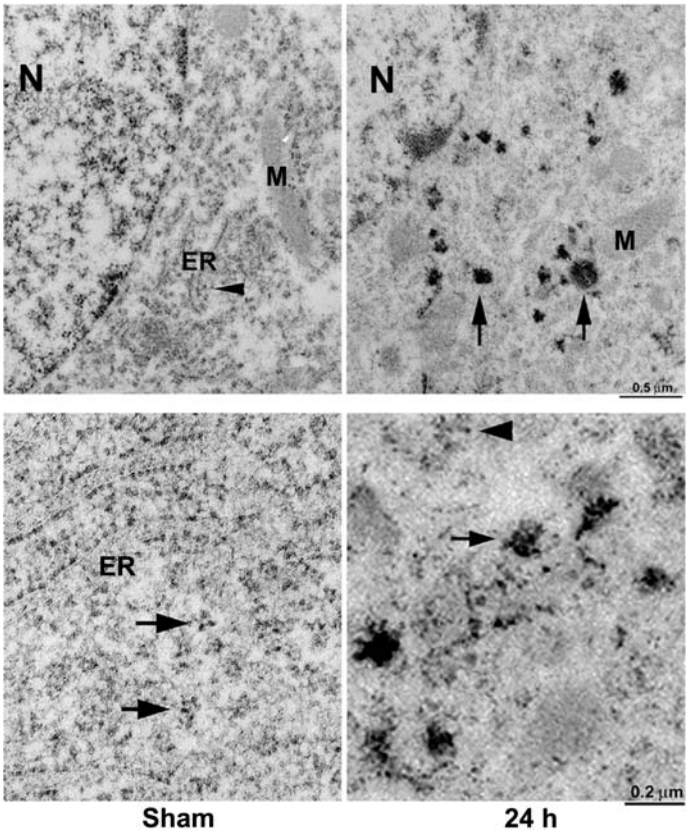


Figure 7-4

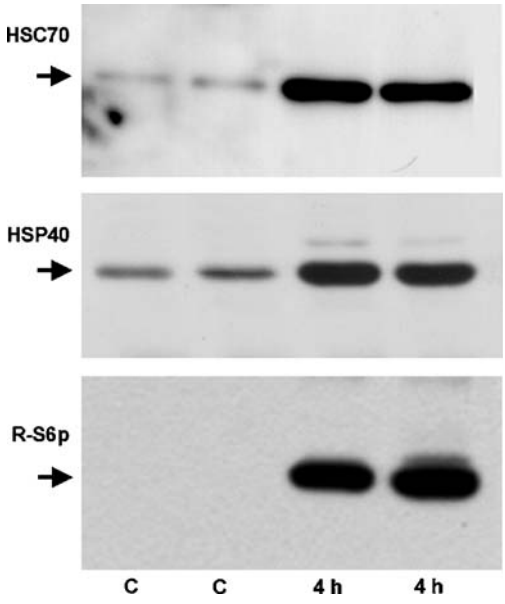
Electron micrographs of EPTA-stained CA1 pyramidal neurons from a sham-operated control rat and a rat subjected to 15 min of cerebral ischemia followed by 24 h of reperfusion. The sections were further counterstained postembedding with a ribosomal selective staining method. Although membranes were not directly visible in EPTA-EM sections due to lipid extraction in this staining protocol, the ER and mitochondria (M) were often visible in negative contrast. *Upper panel*: the ER, mitochondria (M), nucleus (N), and ribosomal rosettes (*arrowhead*) are normally distributed in sham-operated CA1 neurons (Sham, *upper panel*). After ischemia, scattered ribosomes appear clumped into large aggregates (*arrows*), resulting in the cytoplasmic space appearing ''emptier''. *Lower panel*: higher magnification of the ribosomal areas. Ribosomal rosettes (*arrows*) and the ER-associated ribosomes are normally distributed in sham-operated control CA1 neurons. After ischemia, ribosomes are abnormally clumped into large aggregates (*arrow*) but some single ribosomes are still seen in cytoplasm (*arrowhead*). Scale bars: 0.5 μm in the *upper panel* and 0.2 μm in the *lower panel*



ribosomes at 30 min reperfusion (Kirino et al., 1984; Deshpande et al., 1992; Tomimoto and Yanagihara, 1992). During 4 and 24 h of reperfusion in CA1 neurons, however, most ribosomes do not revert to the rosette polyribosomal structure, but rather remain either single or appear as disordered large clumps (aggregates) that are stained with osmium–uranium–lead under electron microscopy (*Figure 7-3*). These ribosome-like aggregates are highly counterstained with a ribosomal selective staining method in CA1 neurons after ischemia (*Figure 7-4*). As a result, postischemic cytoplasm appears "emptier" (*Figures 7-3* and *7-4*). On Western blots, ribosomal S6 protein, HSC70, and HSP40 are highly accumulated in the detergent-insoluble protein aggregate-containing fraction from CA1 neurons after ischemia (*Figure 7-5*). Aggregated HSC70 loses chaperone function in vivo (Angelidis et al., 1999). This series of events depicts a

Figure 7-5

Immunoblots of a ribosomal S6 protein, HSC70, and HSP40 in the detergent-insoluble protein aggregate containing fraction after brain ischemia. The CA1 regions were dissected from sham-operated control rats (Ctr) and rats subjected to 15 min of cerebral ischemia followed by 4 h of reperfusion. Two separate samples in each experimental group were run in parallel on SDS-PAGE. The blots were labeled with antibodies against S6, HSC70, and HSP40 proteins and visualized with the ECL system



possible scenario such that newly synthesized polypeptides together with their associated ribosomes and folding assistant proteins may be abnormally aggregated in ischemic vulnerable neurons. It appears likely that nascent polypeptides on ribosomes cannot fold or degrade due to the disabilities of chaperoning and degradation machinery after ATP-depletion and changes in cell homeostasis following ischemia. Consequently, unprocessed nascent polypeptides, together with their associated ribosomes, chaperones and cochaperones, are stacked and gradually aggregated during the postischemic phase. Protein aggregation is virtually an irreversible process. Therefore, irreversible aggregation of protein synthesis and folding machinery may explain persistent inhibition of protein synthesis in CA1 neurons after ischemia (Cooper et al., 1967; Mies et al., 1991; Hossmann et al., 1993; Hu and Wieloch, 1993).

4 Concluding Remarks

Cotranslational protein aggregation in ischemic vulnerable neurons may represent a new mechanism underlying delayed neuronal death after an episode of ischemia. An ischemia-induced cascade of energy failure, intracellular calcium overload, overproduction of ROS, and acidosis cumulatively disables and damages ATP-dependent protein quality control machinery for cotranslational folding and folding-coupled degradation after brain ischemia. As a result, nascent polypeptides are unable to fold correctly. Consequently, aberrant nascent polypeptides together with their associated chaperones and ribosomes may be irreversibly aggregated with each other or with other subcellular structures. This cotranslational protein aggregation may demolish ribosomal protein synthesis machinery in ischemic vulnerable neurons. Cotranslational protein aggregation-induced damage accumulates over time, and when it reaches a critical degree, may

lead to delayed cell death after ischemia. This hypothesis seems consistent with several existing ones: (1) energy failure and loss of ionic homeostasis seems necessary to induce neuronal damage after ischemia. Both depletion of ATP and loss of ionic homeostasis disable cotranslational folding machinery and cause overproduction of toxic abnormal proteins in cells (Hartl and Hayer-Hartl, 2002); (2) permanent inhibition of protein synthesis is the most accurate indicator for neuronal death after ischemia (Cooper et al., 1977; Mies et al., 1991; Hossmann et al., 1993; Hu and Wieloch, 1993). Abnormal aggregation of ribosomal proteins results in permanent inhibition of protein synthesis after brain ischemia; (3) free ubiquitin is permanently depleted in ischemic vulnerable neurons (Magnusson and Wieloch; 1989; Morimoto et al., 1996; Hu et al., 2000, 2001). Abnormal proteins accumulating in neurons deplete intracellular free ubiquitin to form ubi-proteins after ischemia; (4) expression of molecular chaperones before ischemia protects neurons against ischemia (Nowak et al., 1985; Sharp et al., 1999; Yenari et al., 2001; Gifford et al., 2004). Molecular chaperones may directly neutralize accumulated unfolded proteins and assist protein degradation (Gifford et al., 2004). However, molecular chaperones are less effective in dissociation of protein aggregates, and protein aggregation is virtually irreversible (Hu et al., 2000); (5) dark substances or materials were observed previously in CA1 neurons after ischemia (Kirino et al., 1984; Deshpande et al., 1992; Tomimoto and Yanagihara, 1992). These dark materials are likely to be protein aggregates (Hu et al., 2000, 2001). For the question about whether induction of HSP70 by ischemia itself is neuroprotective, recent evidence suggests that unfolded proteins are already irreversibly aggregated at 2–4 h of reperfusion (Hu et al., 2000, 2001), but HSP70 is not induced until 4–24 h of reperfusion. Therefore, induction of HSP70 by ischemia itself may be too late to prevent protein aggregation after ischemia. It should also be mentioned that it is still a subject of debate whether protein aggregates per se are toxic to cells. However, it is generally held that protein aggregation reflects cellular overload of toxic abnormal proteins that may exert highly toxic effects on neurons before their aggregation (Ross et al., 1999). Our studies support the idea that clumping of protein aggregates on cellular machinery such as protein synthesis machinery in living neurons for an extended period of time will eventually lead to neuronal death after brain ischemia (Hu et al., 2004).

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8 Superoxide Dismutases in Stroke

C. M. Maier · P. H. Chan

Abstract: Oxygen is essential for the survival of aerobic organisms. However, as it is capable of oxidizing any biological molecule, oxygen also has the capacity to cause widespread cellular damage and even death. This is because each oxygen atom has one unpaired electron in its outer shell, making atomic oxygen a free radical. A growing body of evidence suggests that many of the causes and the consequences of ischemic injury to the brain are enhanced by reactions involving oxygen-derived free radicals such as superoxide. This process, known as oxidative stress-induced damage, results from an imbalance between oxidant production and the antioxidant capacity of the affected cells. Thus, understanding the role of endogenous antioxidant defense systems is critical for the development of therapeutic strategies aimed at prevention and treatment of cerebral ischemic injury. This chapter summarizes the cellular and molecular mechanisms underlying oxidative tissue damage in the acute, subacute, and chronic stages of stroke, as well as the link between oxidative stress and certain stroke risk factors. Particular focus is placed on the role of superoxide dismutases in stroke etiology and evolution. Experimental evidence from laboratory animals that either overexpress or are deficient in antioxidant enzyme/protein levels is reviewed. Finally, the potential use of antioxidants for stroke treatment is discussed.

List of Abbreviations: SOD, Superoxide Dismutase; SODs, Superoxide Dismutases

1 Introduction

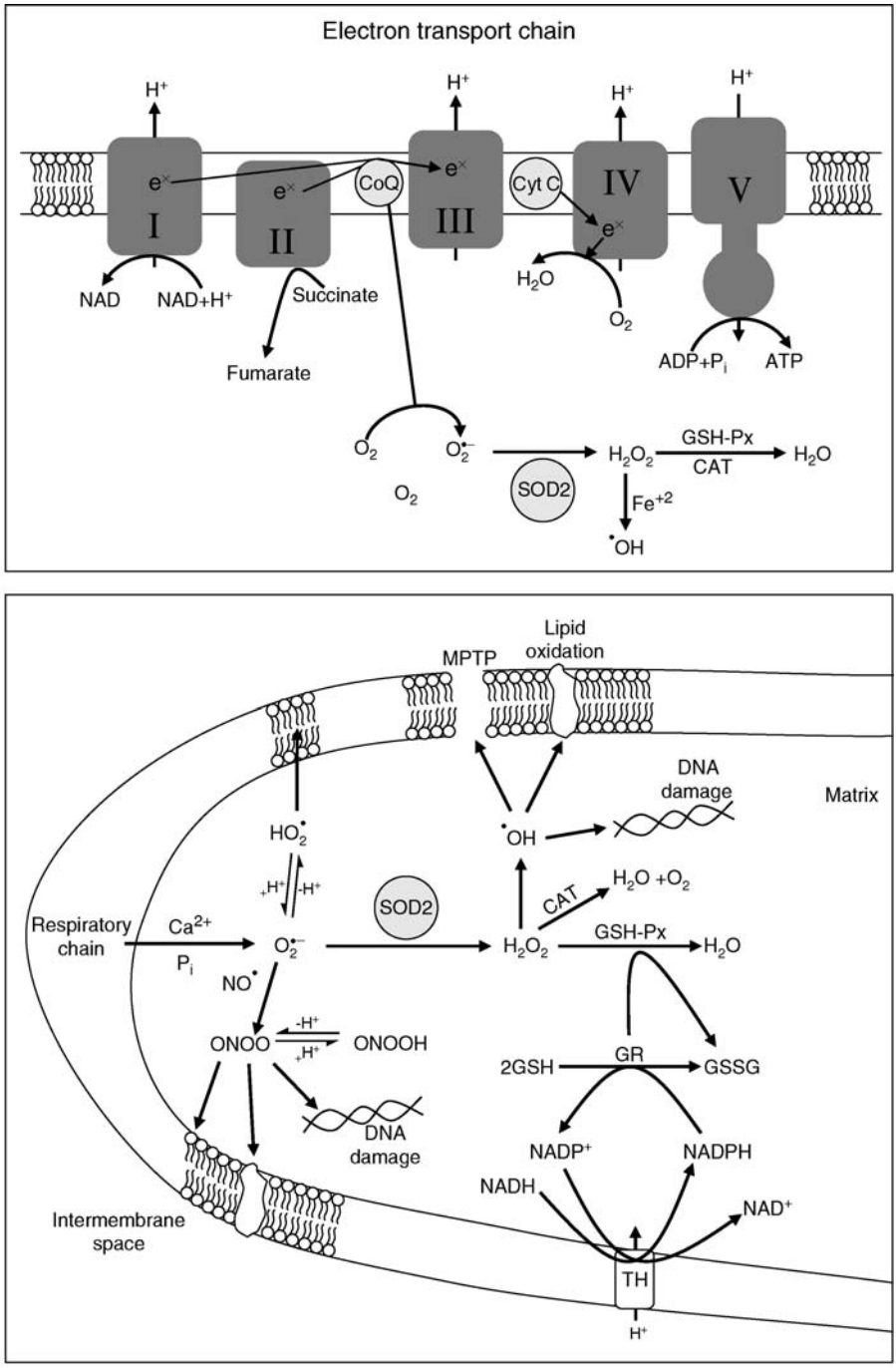
Oxygen is essential to fuel energy production in mitochondria and is, therefore, indispensable for the survival of aerobic organisms. The dioxygen molecule (O_2) is routinely reduced by the mitochondrial electron transport chain to produce water through a well-regulated and relatively safe process. However, successive single-electron reduction reactions of molecular oxygen can also generate reactive intermediates. These reactive oxygen species (ROS) include oxygen-free radicals as well as nonradical oxygen derivatives that are involved in the production of oxygen radicals through a variety of chemical reactions (for reviews see Imlay, 2003; Andreyev et al., 2005).

Superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) are among the ROS produced by the reduction of oxygen molecules. Hydrogen peroxide is often generated in cells via the production of superoxide, known as a dismutation reaction, which can take place either spontaneously (albeit very slowly) or can be catalyzed by the enzyme superoxide dismutase (SOD). Hydrogen peroxide is then scavenged by catalase or glutathione peroxidase (GSH-Px) at the expense of glutathione (GSH) and reduced to water and oxygen. GSH is generated from oxidized GSH by GSH reductase in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH). In the presence of transition metal ions, H_2O_2 can produce $^{\bullet}OH$, a highly reactive chemical species capable of attacking phospholipids and nucleic acids, thereby leading to lipid peroxidation and DNA damage (Phillis, 1994). Another powerful ROS is peroxynitrite ($ONOO^-$), a high-energy oxidant derived from superoxide and nitric oxide (NO) (*Figure 8-1*). Peroxynitrite can inhibit the function of SOD (MacMillan-Crow et al., 1996) and the mitochondrial respiratory chain (Brorson et al., 1999), thus leading to a positive feedback cycle of cytotoxic

Figure 8-1

(a) The mammalian oxidative phosphorylation system consists of five membrane-bound protein complexes: (I) NADH-ubiquitol oxidoreductase that has at least six iron–sulfur clusters which can be subject to superoxide-mediated inactivation (Ohnishi, 1998), (II) succinate-ubiquitol oxidoreductase that has three iron–sulfur centers, (III) ubiquitol-cytochrome *c* oxidoreductase, (IV) cytochrome *c* oxidase, (V) and ATP synthase. During hypoxic insult, cytosolic protons increase leading to increased membrane potential. A high mitochondrial membrane potential results in inhibition of electron transport by the respiratory chain and increased half-life of intermediates capable of reducing O_2 to superoxide anion ($O_2^{\bullet -}$). (b) When $O_2^{\bullet -}$ generation increases together with intracellular Ca^{2+} buildup and/or hydrogen peroxide (H_2O_2), removal pathways are inactivated, H_2O_2 accumulates. H_2O_2 is converted by one pathway to H_2O and O_2 by catalase (CAT) in peroxisomes and by glutathione peroxidase (GSH-Px) in the cytoplasm, at the expense of reduced glutathione (GSH), leading to the formation of oxidized glutathione disulphide (GSSG), which can be recycled back to GSH by glutathione

reductase (GR). In the presence of Fe^{2+}, H_2O_2 can generate the highly reactive hydroxyl radical ($^{\bullet}OH$), which in turn can oxidize thiol groups in the inner mitochondrial membrane, leading to assembly and opening of a large conductance channel known as the mitochondrial permeability transition pore (MPTP). Hydroxyl radicals can also promote membrane permeabilization through lipid oxidation (modified from Kowaltowski et al., 2001)



increases in $O_2^{\bullet-}$ and $ONOO^{\bullet-}$ formation. Interestingly, the rate of reaction between $O_2^{\bullet-}$ and NO is considered to be the fastest in biological systems (2×10^{10} M^{-1} s^{-1}) (Kissner et al., 1998), which explains the need for high SOD concentrations (10^6 times higher than that of $O_2^{\bullet-}$) so that this enzyme can compete effectively for $O_2^{\bullet-}$ when NO is present (Johnson and Giulivi, 2005).

Aside from being a source of H_2O_2, superoxide serves as a reducing agent of transition metal ions. Thus, the significance of this free radical is further highlighted by the fact that superoxide can react with iron sulfur centers commonly found in mitochondria and can lead to the inactivation of a variety of enzymes that are critical for cell survival and function (Hausladen and Fridovich, 1994; Vasquez-Vivar et al., 2000). The major source of $O_2^{\bullet-}$ in cells is electron "leakage" from two distinct sources: (1) the electron transport chain in mitochondria, which generates most of the cellular energy via oxidative phosphorylation (between 0.4% and 4% of the oxygen consumed is reduced to form superoxide during this process) (Melov et al., 1999; Chan, 2004), and (2) the endoplasmic reticulum, a principal site for the synthesis of membrane lipids, membrane and secretory proteins, intracellular calcium homeostasis, and cell death signal activation (Cullinan and Diehl, 2005; Hayashi et al., 2005). Superoxide is also generated by NADPH oxidases, xanthine oxidase, cyclooxygenase, and flavin oxidases, as well as by autooxidation of certain compounds like ascorbic acid (vitamin C), thiols (GSH and cysteine), adrenaline, and flavin coenzymes (Cheeseman and Slater, 1993).

Superoxide anion production along with SOD expression and activity have been implicated in several pathological states in the central nervous system (CNS) (Maier and Chan, 2002), including ischemia-reperfusion injury (Imaizumi et al., 1990; Kinouchi et al., 1991; Uyama et al., 1992; Kim et al., 2005), vasospasm after subarachnoid hemorrhage (Kajita et al., 1994; Kamii et al., 1999; Aladag et al., 2003; Watanabe et al., 2003; Kaynar et al., 2005; Zheng et al., 2005), atherosclerosis (Miller et al., 1998; Landmesser et al., 2005; Tasaki et al., 2005), and meningitis (Pfister et al., 1992; Schaper et al., 2002; Cemek et al., 2005).

Despite their toxic potential, ROS are purposefully generated by animal cells and serve important physiological functions. Some enzymes, for example, use free radicals at their active site during catalysis (Adams and Odunze, 1991). Activated phagocytes deliberately generate superoxide as part of their bactericidal role, and the inability to produce ROS, such as in granulomatous disease, results in chronic infectious disorders as well as cognitive deficits (Curnutte et al., 1974; Pao et al., 2004). ROS also play important roles in cell proliferation, differentiation, and signaling (Chan, 2004). This is evidenced by the fact that growth factors and cytokines are capable of generating ROS in a number of different cell types, including neurons, glia, and vascular cells (Irani, 2000). Furthermore, antioxidants can block specific growth factor- and/or cytokine-activated signaling events and physiological effects (Suzuki et al., 1997; Thannickal and Fanburg, 2000). Intracellular ROS have also been implicated in neuronal differentiation induced by NGF (Suzukawa et al., 2000) and hypoxic mimetic agents (Kotake-Nara et al., 2005), as well as in the development of the neuronal phenotype (Tsatmali et al., 2005).

To survive in an atmosphere containing 20% oxygen and the inevitable (and physiologically necessary) generation of ROS, aerobic organisms have developed an array of antioxidant defenses including enzymes to decompose peroxidases (e.g., catalase and GSH-Px), proteins to sequester transition metals, and antioxidant enzymes such as superoxide dismutases whose main role is to intercept and inactivate ROS. The goal of this defense system is to minimize the destructive actions of ROS without compromising normal and essential biological processes. However, when the endogenous antioxidant systems become overwhelmed, proteins, lipids, DNA, and other macromolecules become the targets of oxidative modification leading to cell dysfunction, tissue damage and death by apoptosis or necrosis. Such is the case in ischemic brain injury (stroke), the consequences of which are enhanced by free radical reactions.

The interruption of cerebral blood flow and ensuing ischemia results in rapid loss of high-energy phosphate compounds (i.e., adenosine triphosphate [ATP]), generalized membrane depolarization, and massive release of excitatory neurotransmitters such as glutamate. Oxidative damage, impaired energy metabolism, and mitochondrial dysfunction are all closely related to excitotoxicity. For example, extracellular accumulation of glutamate leads to activation of the *N*-methyl-D-aspartate (NMDA) receptor. Superoxide generation has been associated with NMDA receptor activation, which leads to stimulation of nitric oxide synthase (NOS) and the generation of NO as well as $O_2^{\bullet-}$ and H_2O_2 (Lewen et al., 2000). If blood flow can be reestablished (reperfusion) following an ischemic stroke, a second wave of oxidative stress occurs.

In the case of reversible middle cerebral artery occlusion (MCAO), there is a burst-like pattern of ROS production within the first 10–15 min of reperfusion (Peters et al., 1998). Expression of SOD1 and SOD2 increases following an ischemic insult (Liu et al., 1993; Matsuyama et al., 1993; Maier et al., 2002), but their activity is differentially affected by the intensity and duration of the ischemic challenge.

The brain, which consumes nearly 20% of inspired oxygen, is especially susceptible to oxidative damage for several reasons. First, the brain has a high content of polyunsaturated fatty acids that are particularly vulnerable to free radical attacks. Second, activities of antioxidant defense systems in the brain are only moderate, and several areas of the brain are rich in intracellular iron, which is readily released by cell injury. The brain is also rich in epinephrine, norepinephrine, and dopamine, all of which react to form superoxide, and these oxidation reactions are accelerated by iron ions. Not surprisingly then, ROS have been implicated in the pathogenesis of cerebral infarction following both global and focal ischemia as well as in brain trauma and other cerebrovascular disorders. Several research strategies have been used to examine the exact role of ROS in stroke, including measurement of free radical reaction products, in situ detection of oxygen radical production, and use of pharmacological agents and antioxidants to establish a correlation between drug administration and injury development. Progress in the field has also been substantially aided by the availability of genetically manipulated animals deficient in or overexpressing enzymes/proteins in the free radical pathways, thus allowing examination of the mechanisms that underlie stroke etiology and disease progression.

Administration of SOD and overexpression of SOD protein have been used as therapeutic strategies to treat hypoxic/ischemic cerebral injury with very profound protective effects in experimental animals. As with most neuroprotective strategies, free radical scavengers have had disappointing results in clinical trials. However, recent studies in humans have renewed hope that these agents may indeed be effective in stroke treatment (Green and Ashwood, 2005). The vast and multifaceted field of stroke prevents us from covering all aspects of free-radical biology in cerebral ischemia. Instead, this chapter focuses on the specific contribution of superoxide and SOD in stroke risk factors and stroke evolution (acute, subacute, and chronic phases). Some of the research studies in animals that are either deficient in or that overexpress SOD isoforms are highlighted and the current therapeutic strategies utilizing SOD are also discussed.

2 SOD Isoenzymes

SOD was first identified in 1969 by McCord and Fridovich (McCord and Fridovich, 1969) and has since become the target of intense investigation in a variety of pathological conditions. There are three types of SOD isoenzymes identified so far in mammals, and they are characterized by their prosthetic metal ion and cellular localization. The zinc-containing SOD (Cu/Zn-SOD or SOD1) is the most abundant and ubiquitous isoform, with an in vivo concentration estimated to be approximately 10 μM (Rae et al., 1999) and with a plasma half-life of approximately 6–10 min due to rapid renal clearance (Bayati et al., 1988; Beckman et al., 2002). This constitutively expressed SOD is a relatively small dimer consisting of 153 amino acids that exists intracellularly in the cytosol, nucleus, peroxisomes, and mitochondrial intermembrane space (Okado-Matsumoto and Fridovich, 2001). Superoxide is attracted to the active site of SOD containing the copper, but the zinc atom also plays an important role in SOD1. Indeed, loss of zinc greatly changes the properties of SOD1 making it toxic to neurons (Beckman et al., 2002). SOD1 catalyzes the reaction of $ONOO^-$ with tyrosine residues resulting in protein nitrosylation. The inducible manganese-containing SOD (MnSOD or SOD2) is a 24-kDa isoenzyme located in mitochondria with a half-life of 4–20 h, depending on species (Baret et al., 1984). Copper- and zinc-containing extracellular SOD (EC-SOD, SOD3) is located mainly in the extracellular space and has a slow clearance, with a half-life of approximately 10 h in the rabbit (Karlsson and Marklund, 1988).

Cu/Zn-SOD and MnSOD are differentially distributed in the neuronal and glial compartments. SOD1 is located primarily in astrocytes throughout the CNS, but the enzyme is also detectable in neurons, while SOD2 is localized predominantly in neurons throughout the brain and spinal cord (Lindenau et al., 2000). SOD2 is observed in astroglial cells to a much lesser degree than in neurons, unless the tissue has undergone an insult and the process of gliosis is under way (Maier et al., 2002). The same is true for the expression of

SOD1 and SOD2 in microglial cells, oligodendrocytes, and endothelial cells where immunoreactivity for both isoenzymes is low under normal physiological conditions. This may help explain why oligodendroglia are particularly vulnerable to oxidative stress (Husain and Juurlink, 1995). Microglial cells, on the other hand, are more resistant to oxidative challenges since they possess high amounts of GSH-Px and are thus able to detoxify H_2O_2 (Lindenau et al., 2000). Astrocytes are also able to upregulate GSH-Px expression, while neurons lack this ability.

EC-SOD is the only SOD isoform that is released from cells in the extracellular space, and it is found in interstitial spaces of tissues and in extracellular fluids (plasma, lymph, cerebrospinal fluid and synovial fluid) as well as in cerebral vessels (Marklund, 1982). It is characterized by a heparin-binding domain and can bind to heparan sulfate proteoglycans on the cell surface. Removal of the heparin-binding domain affects the affinity of EC-SOD for and its distribution to the extracellular matrix in which it is secreted (Enghild et al., 1999). EC-SOD, considered a regulator of NO bioavailability (Pineda et al., 2001), is highly expressed in vascular smooth muscles and may play a key role in cerebral vascular biology and vasomotor dysfunction (Nakane et al., 2001; Tasaki et al., 2005). EC-SOD expression is increased by NO as well as by angiotensin II (Ang II), endothelin, and heparin and is reduced by growth factors. Interestingly, atherosclerosis depresses EC-SOD gene expression, whereas treatment with Ang II type 1 (AT1) receptor antagonists as well as angiotensin-converting enzyme (ACE) inhibitors increases EC-SOD expression levels (Hornig et al., 2001; Strehlow et al., 2003). In fact, expression of antioxidant enzymes in general can be altered by hormones such as Ang II, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β.

3 Elucidating the Role of SOD Isoenzymes

Cellular signaling involves many cytoplasmic protein kinase cascades that form pathways connecting extracellular stimuli (e.g., cytokines and growth factors) to the nucleus, resulting in transcription and synthesis of proteins. These signaling pathways are very sensitive to the cellular redox status and respond to many oxidative stresses. Several cell death and cell survival signaling pathways are activated following an ischemic stroke, and these have been extensively reviewed elsewhere (Martindale and Holbrook, 2002; Chan, 2004; Taylor and Crack, 2004). Here, we review a few of the animal studies that highlight the importance of SOD in stroke-related cell-signaling mechanisms. To aid the reader, a schematic diagram is included summarizing some of the life/death signaling in ischemic neurons, with particular focus on the intrinsic mitochondria-dependent apoptotic pathway (*Figure 8-2*). It is important to keep in mind that death/survival signaling pathways can be differentially activated and regulated depending on the cell type, the intensity and duration of the ischemic insult, the phase of the disease condition (e.g., acute versus chronic stroke), and the developmental stage of the animal. Furthermore, the combined effects of individual signaling pathways can be either additive or antagonistic. Ultimately, these carefully orchestrated cellular signaling pathways lead to changes in gene expression that influence the fate of the cell. Understanding how these pathways work under normal and pathological conditions is critical in order to identify appropriate therapeutic targets.

Numerous studies using genetically manipulated animals that either overexpress or are deficient in SOD isoforms have helped elucidate the role of these antioxidants in ischemic stroke (for review see Saito et al., 2005). For example, overexpression of SOD1 in adult animals (SOD1 transgenics, SOD1-TGs) has been shown to result in significant reductions in infarct volume following focal cerebral ischemia as well as following global insults (Kinouchi et al., 1991; Chan et al., 1994, 1998; Murakami et al., 1997; Noshita et al., 2002; Sugawara et al., 2002). The neuroprotection observed in SOD1-TGs is due, in part, to a blockade of the mitochondrial pathway of apoptosis that involves cytochrome *c*, second mitochondria-derived activator of caspase (Smac), and Omi release, caspase activation, binding of Smac and inhibitor-of-apoptosis protein (IAP), poly(ADP-ribose)polymerase (PARP) inactivation and DNA fragmentation (Sugawara et al., 2002; Saito et al., 2004a, 2005b), and can be observed in neurons as well as in astrocytes (Wang et al., 2005). SOD1 overexpression may also attenuate the calcium-dependent MPT pore opening or the voltage-dependent anion channel-mediated mitochondrial permeability by directly or indirectly reducing mitochondrial superoxide production (Madesh and Hajnoczky, 2001). Overexpression of SOD1

has also been shown to be protective in other aspects of the apoptotic process. Following transient MCAO, there is degradation and ubiquitylation of the tumor suppressor gene p53, which plays an important role in the regulation of apoptosis through transcriptional activation of cell cycle control. Saito et al. (2005b) have recently shown that SOD1 inhibits the accumulation of ubiquitylated p53 and facilitates p53 degradation by promoting the MDM2 pathway and the ubiquitin-proteasome system, which are critical for p53 ubiquitylation.

However, SOD1 overexpression is not always desirable. Neonatal SOD1-TG mice, for example, have increased neuronal injury following hypoxia-ischemia due to developmentally downregulated GSH-Px activity (Ditelberg et al., 1996; Fullerton et al., 1998). On the other hand, when the constitutively expressed SOD1 is absent, as in the case of SOD1 knockout (KO) mice, the result is increased cell death and edema following transient focal and global ischemia due to exacerbation of the cell death signaling pathways (Kondo et al., 1997a, b; Kawase et al., 1999; Saito et al., 2005b). A 50% reduction in SOD2 activity has similar damaging effects. Heterozygous SOD2-KO mice have increased infarct volumes following permanent MCAO (Murakami et al., 1998), as well as increased mitochondrial cytochrome *c* release and subsequent DNA fragmentation (Fujimura et al., 1999). Interestingly, SOD1 overexpression in SOD2-KO mice results in attenuation of DNA fragmentation and lesion volume following striatal injection of a mitochondrial toxin (Kim and Chan, 2002). In addition to increased infarcts, reperfusion following a mild focal ischemic insult results in delayed blood–brain barrier (BBB) damage and increased hemorrhage rates in SOD2-KO animals (Maier et al., 2006). These adverse consequences are thought to result from ROS-induced activation of matrix metalloproteinases (MMPs) in vascular endothelial cells and subsequent damage to specific tight junction transmembrane proteins. Complete inactivation of the sod2 gene results in a neonatal lethal phenotype (Li et al., 1995), lending further support to the notion that the toxicity of mitochondrial ROS is particularly deleterious. A two to threefold increase in SOD2 activity results in neuronal protection against oxidative stress induced by transient MCAO (Keller et al., 1998) and attenuation of reperfusion-related vascular damage (Maier et al., 2006); however, larger increases in SOD2 activity are potentially detrimental (Kowald and Klipp, 2004). Overexpression of EC-SOD also provides protection against focal and global cerebral ischemia, while its removal or limited activity amplifies the damage (Sheng et al., 1999a, b, 2000).

Figure 8-2

Mitochondria are the primary site of action of the Bcl-2 protein family, which consists of both proapoptotic (e.g., Bid, Bax, Bad,) and antiapoptotic (Bcl-2, Bcl-x_L) members. The precise molecular mechanism by which Bcl-2 family proteins protect against or induce mitochondrial damage is still controversial, but a growing body of evidence suggests that permeabilization of the mitochondrial membrane and the release of mitochondrial proteins are key steps in this process. This schematic depicts a model in which oxidative stress and increased intracellular calcium levels trigger the mitochondrial release of apoptogenic factors such as cytochrome *c* (Cyt c), the second mitochondria-derived activator of caspase (Smac), apoptosis-inducing factor (AIF), the serine protein Omi (also known as high-temperature requirement A, HtrA, or Omi/HtrA2) and endonuclease G. Cytochrome *c* and Smac induce cellular damage by amplifying caspase-mediated proteolysis, whereas caspase-independent pathways include AIF, Omi/HtrA2, and endonuclease G. Cytochrome *c* interacts with Apaf-1 to form the apoptosome, which results in the initiation of the proteolytic cascade that culminates in apoptosis. Some proteins, such as the inhibitor-of-apoptosis proteins (IAPs), can prevent caspase activation in the cytosol. Smac can bind IAPs, thus promoting caspase activation. Life and death signaling in ischemic neurons involving mitochondria and the phosphatidylinositol 3-kinase-(PI3-K)/Akt/Bad pathway is also illustrated. Serine/threonine kinases such as Akt (also known as kinase B) are key regulators of survival after cerebral ischemia. Akt is a major downstream target of PI3-K and can phosphorylate Bad, which results in blockade of Bax-mediated channel formation in the mitochondrial membrane, and thus, inhibition of cytochrome *c* release. Akt also inhibits caspase-9 activity and can translocate into the nucleus where it inactivates proapoptotic transcription factors. In addition, Akt can phosphorylate proline-rich Akt substrate (PRAS), thought to play a role in nerve growth factor-induced neuroprotection (modified from Saito et al., 2005)

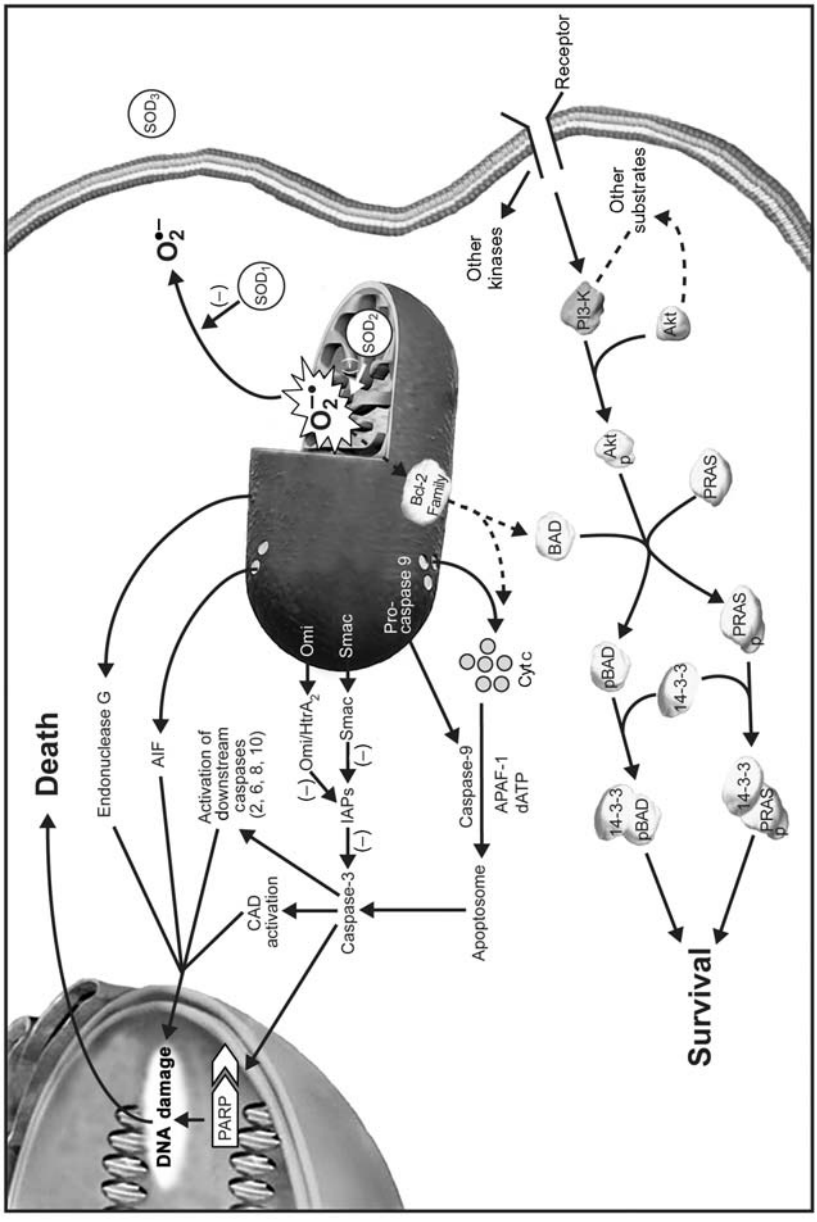


Figure 8-2 (continued)

In the past, most experimental stroke research on ROS and SOD isoenzymes has focused on cell death mechanisms. In recent years, however, increasing attention has been paid to the involvement of SODs in cell survival signaling pathways. As previously mentioned, many cellular cytoplasmic protein kinase cascades are activated in response to external stimuli and oxidative stress. These kinases include mitogen-activated protein kinase (MAPK) family members (e.g., extracellular signal-regulated kinases, ERK1/2), phosphatidylinositol 3-kinase (PI3K)/Akt, and protein kinase C (PKC) members (Saito et al., 2005b). The PI3K/Akt axis, for example, which is utilized by various cell types for inhibition of apoptosis and cellular survival, has been shown to upregulate SOD1 expression via the activation of nuclear factor-κB (NF-κB), suggesting that activation of this pathway might reinforce the antioxidant cell capacity and provide protection against oxidant damage via the upregulation of antioxidant genes (Rojo et al., 2004). Oxidative stress has also been shown to modulate the expression of phosphorylated proline-rich Akt substrate (pPRAS), which is downstream of PI3-K, and the binding of pPRAS/phosphorylated Akt (pPRAS/pAkt) to 14-3-3 (pPRAS/14-3-3). PRAS phosphorylation and its interaction with pAkt and 14-3-3 might play an important role in neuroprotection mediated by nerve growth factor (Saito et al., 2004b, 2005b). These findings could ultimately lead to the development of therapeutic approaches aiming not only at reducing neuronal cell death but also at improving neuronal survival and regeneration following a stroke.

4 Stroke Risk Factors, Superoxide, and SOD Isoenzymes

There are many modifiable and nonmodifiable risk factors for stroke. Here, we summarize a limited number of studies on common risk factors that have been associated with the production of oxygen radicals and highlight the role of SODs in cerebrovascular disorders.

4.1 Hypertension

Hypertension is the single most important risk factor for stroke. Elevated blood pressure promotes atherosclerosis and exerts abnormal pressure on blood vessel walls, which can lead to vessel injury and eventual rupture (for review see de Nigris et al., 2003), although the exact mechanisms through which oxygen radicals influence blood pressure have not been fully elucidated. ROS contribute to the pathogenesis of hypertension by influencing vascular function and structure. Superoxide anion production is increased in experimental models of hypertension (Kerr et al., 1999; Somers et al., 2000) as well as in human hypertension (Touyz and Schiffrin, 2001). Recently, Campese et al. (2004) have shown that tempol, a SOD mimetic, reduces central sympathetic nervous system activity as well as renal sympathetic nerve activity, further highlighting the role of superoxide in the modulation of blood pressure.

The study of hypertension and its effects on vessel structure has been greatly aided by the development of genetic animal models such as the spontaneously hypertensive rat (SHR) and the stroke-prone SHR. Studies of these animals have revealed that hypertension leads to alterations in BBB tight junctions and in endothelial cell polarity, as well as alterations in gene expression in the microvasculature (Kirsch et al., 2001). Furthermore, studies performed in SHR suggest that enhanced production of NADPH oxidase-dependent $O_2^{\bullet -}$ contributes to endothelial dysfunction and vascular hypertrophy (Zalba et al., 2000). Interestingly, SHR have reduced SOD and GSH-Px activity compared with normotensive animals (Hector Polizio and Pena, 2005), and this reduction in enzymatic activity can also be induced by oxidized low-density lipoprotein, a key mediator of atherogenesis (Rosenblat and Aviram, 1998; de Nigris et al., 2003).

Aside from altering the structure of blood vessels, hypertension also disrupts the regulation of cerebral blood flow (CBF), which may contribute to cognitive impairment and microvascular ischemic brain injury that are associated with chronically elevated blood pressure (Droste et al., 2003; Iadecola and Gorelick, 2004). Angiotensin II has emerged as a critical factor in the deleterious cerebrovascular effects of hypertension. For example, Ang II impairs the increase in CBF produced by neural activity (known as functional hyperemia) through NADPH oxidase-derived radicals (Kazama et al., 2004). It has been recently shown that superoxide is involved in the Ang II–stimulated influx of extracellular Ca^{2+}in neural cells, lending further

support for a potential intracellular signaling mechanism involved in Ang II-mediated oxidant regulation of central neural control of blood pressure (Zimmerman et al., 2005). Work on stroke-prone SHR has also shown that AT1 receptor-mediated NADPH oxidase-generated ROS, endothelial NOS, and Akt might be crucial determinants for the vascular smooth muscle cell (VSMC) phenotype in hypertension (Kawahara et al., 2005). Treatment with AT1 receptor antagonists or ACE inhibitors inhibits vascular remodeling and reduces ROS in stroke-prone SHR via not only a reduction in NADPH oxidase but also an upregulation of SOD1 (Tanaka et al., 2005).

4.2 Hyperhomocysteinemia

Homocysteine (Hcy) is a sulfur-containing amino acid that is formed exclusively upon demethylation of the essential amino acid methionine. Hcy is metabolized either through remethylation or transsulfuration pathways and is nutritionally regulated (Loscalzo, 1996). An elevated plasma Hcy level is denoted hyperhomocysteinemia (HHcy), an important and independent risk factor for stroke and cardiovascular diseases (Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis, 2002). Hyperhomocysteinemia can accelerate the development of atherosclerosis and is also a potent inducer of endothelial dysfunction, particularly in small vessels such as cerebral arterioles (Wilson and Lentz, 2005). Accumulating evidence suggests that oxidative stress mechanisms may mediate the effects of homocysteine-induced vascular injury. Like most thiol molecules, homocysteine can undergo auto-oxidation in the presence of transition metal catalysts and molecular oxygen, leading to ROS formation. When paired with copper, for example, Hcy can induce H_2O_2 generation and alterations in the arterial endothelial barrier (Berman and Martin, 1993). Various in vitro studies using vascular tissues have implicated Hcy in abnormal vascular relaxation responses through enhanced intracellular superoxide production, which results in decreased availability of endothelial NO and H_2O_2 formation (Gryglewski et al., 1986; Tawakol et al., 1997; Chambers et al., 1999; Lang et al., 2000; Mosharov et al., 2000; Weiss et al., 2002; Stanger and Weger, 2003). In animal models, both genetic and pharmacological approaches have been used to demonstrate the role of ROS in HHcy-induced endothelial dysfunction. Dayal et al. (2004), for example, showed that tiron, a superoxide scavenger, could restore the impaired dilatation of cerebral arterioles (in response to acetylcholine) in diet-induced hyperhomocysteinemic mice. Moreover, dihydroethidium (DHE) staining for vascular superoxide, which was increased in those animals, was inhibited by apocynin or *N*-ω-nitro-L-arginine methyl ester, implicating NADPH oxidase and NOS as potential sources of superoxide. Increased vascular DHE staining has also been observed in methionine synthase-deficient mice (Dayal et al., 2005), which have defective homocysteine remethylation, and in apoE-deficient animals (which have HHcy-induced accelerated atherosclerosis) fed a high homocysteine diet (Zhou et al., 2004). In humans, hyperhomocysteinemia has been associated with an increased oxidation rate of aminothiols in plasma, which further contributes to increased ROS generation. These effects are amplified by a homocysteine-specific inhibition of SOD and the cellular isoform of GSH-Px (Weiss, 2005). However, the link between oxidative stress and homocysteine-mediated endothelial dysfunction in human patients is still controversial (Chao et al., 2000), perhaps due to the fact that thiols can function both as anti- and prooxidants depending on the location of the oxidative damage occurring to lipoproteins (Perna et al., 2003). Finally, despite HHcy being considered an independent risk factor for cardiovascular disease, lowering homocysteine levels by as much as 30% have not been effective in reducing the risk of subsequent cardiovascular events (NORVIT trial), raising the question of whether high homocysteine levels are simply an epiphenomenon rather than a direct cause of vascular injury.

4.3 Diabetes

The association between diabetes and increased stroke risk stems from the circulatory problems caused by alterations in glucose transport and metabolism. Hyperglycemia stimulates many cellular pathways which result in oxidative stress, including increased production of advanced glycosylated end products, PKC

activation, and polyol pathway flux (Mabley and Soriano, 2005). When hyperglycemia-induced superoxide formation is paired with endothelial NO production (important for the regulation of normal vascular tone), the result is nitrosative stress (via $ONOO^-$ formation) and cellular damage. Insulin-induced hypoglycemia and diabetes-induced hyperglycemia also lead to decreased SOD2 and catalase activity in the brain (Singh et al., 2004). This decrease in enzymatic activity has been postulated to result from ROS-mediated site-specific amino acid modification or by a superoxide-mediated suppression of the sod2 gene. Similar decreases in SOD activity have been obtained in brain tissue from db/db diabetic mice (Makar et al., 1995) and in alloxan-diabetic animals, which also have significant alterations in the metabolism of lipid peroxides and glycolipids (Kumar and Menon, 1993). Thus, hypoglycemia and diabetes-induced hyperglycemia may render brain tissue more vulnerable to free radical attacks and oxidative damage. However, not all studies support this hypothesis. SOD1 and SOD2 protein expression and activity are reportedly increased in streptozotocin (STZ)-induced diabetic rats in a variety of brain regions, including the hippocampus (Huang et al., 1999) and in its sub regions, particularly the dentate gyrus (Grillo et al., 2003). Posttranscriptional upregulation of SOD2 and SOD1 levels has also been observed in rats subjected to permanent focal stroke under acute hyperglycemia (Bemeur et al., 2004), although this may reflect a transient protective response to increased superoxide production. Under chronic diabetic conditions, increases in SOD expression and activity in specific brain regions may be indicative of sustained oxidative stress in those areas. Interestingly, aerobic exercise appears to increase antioxidant defenses in the brain of diabetic but not normoglycemic animals (Ozkaya et al., 2002).

4.4 Other Risk Factors

Additional stroke risk factors include a previous stroke or transient ischemic attack, cardiovascular disease, smoking, elevated lipid levels, stress, male gender, advanced age, and race. The relationship between some of these risk factors and superoxide dismutases is not completely clear, but links are emerging. For example, smoking is known to cause significant oxidative stress—one puff of cigarette smoke contains millions of reactive oxygen and nitrogen species, and tar includes organic compounds such as quinones, which react with O_2 and increase the generation of superoxide. Compounding the problem is the fact that SOD and GSH-Px activities are significantly lower in the erythrocytes of smokers compared with nonsmokers (Orhan et al., 2005). With regards to chronic stress, Grillo et al. (2003) have recently demonstrated that stress reduces SOD1 and SOD2 protein expression in particular sub regions of the rat hippocampus. There are also potentially important interactions between sex steroids and SODs. For example, SOD2 and EC-SOD activity is enhanced by estrogens, possibly by transcriptional mechanisms such as the PI3 kinase pathway (Strehlow et al., 2003). Alterations in antioxidant capacity are also observed in the aging brain, which may predispose certain structures (e.g., hippocampus) to oxidative stress-related damage (Siqueira et al., 2005). Although it is important to isolate specific risk factors to determine their relative contribution to stroke etiology and evolution, many vascular risk factors are interrelated. In fact, recent work by Antier et al. (2004) has shown that, in female rats, there is an age-dependent increase in brain superoxide levels, which is exaggerated in the stroke-prone SHR animals. The excess cortical superoxide levels in these hypertensive rats may be associated with a downregulation of SOD1 but are not related to a decrease in estrogen levels.

As noted earlier, many cardiovascular conditions are also risk factors for stroke. Atrial fibrillation (AF), for example, confers significant risk of stroke caused by embolization of thrombi originating within the left atrial appendage (LAA). Recently, Dudley et al. (2005) have shown that AF is associated with increased superoxide formation in the LAA, with no changes in total SOD activity, which may be an important cause of thrombus formation.

5 Role of SODs in Stroke

Although hemorrhagic and ischemic stroke have different risk factors and pathophysiological mechanisms, ROS-induced oxidative damage is a common denominator. Here, we provide a brief overview of the

relevance of SOD isoenzymes in hemorrhagic stroke and a more detailed summary of the studies that have helped elucidate the role of these antioxidants in the acute, subacute, and chronic phases of ischemic stroke.

5.1 Hemorrhagic Stroke

Following intracerebral (ICH) or subarachnoid (SAH) hemorrhage, there is increased generation of ROS caused, in large part, by the release of iron from lysed red blood cells (RBCs) (for review see Wagner et al., 2003). As previously mentioned, free iron is capable of enhancing $^{\bullet}OH$ production resulting in significant damage to cellular membranes. In addition, ROS-induced DNA damage can be greatly amplified in the presence of free iron (Aruoma et al., 1989). Iron uptake and storage is regulated by iron regulatory proteins (IRP-1 and IRP-2), which are themselves sensitive to oxidative stress. Peroxynitrite and ROS can directly stimulate the translation of iron-binding proteins and iron-transport proteins (Bouton, 1999; Hanson and Leibold, 1999). Interestingly, evidence from in vitro studies suggests that white matter may be more vulnerable to iron-induced ROS production than gray matter (Hall et al., 2000).

Antioxidant treatment or modification of brain SOD levels following experimental hemorrhagic stroke can affect neurologic outcome and reduce neuronal injury. For example, using an SAH model, Matz et al. (2000, 2001) found that DNA damage is inversely related to SOD1 and SOD2 expression. Following intracerebral infusion of lysed RBCs, Wu et al. (2002) found a reduction in SOD1 and SOD2 levels as well as evidence of single-strand DNA breaks within 24 h of the insult, which preceded endonuclease-mediated DNA fragmentation. Evidence for delayed DNA damage has been shown to occur several days after experimental ICH (Matsushita et al., 2000). Together, these studies suggest that antioxidant therapy may be effective for treatment of brain hemorrhage, including both the primary hemorrhage as well as the secondary brain injury following ICH.

SAH leads to an imbalance in the arterial wall vasodilatation and vasoconstriction, which results in cerebral vasospasm. The mechanisms involved include scavenging of NO by superoxide and release/activation of various vasoactive substances such as biogenic amines, eicosanoids, and endothelins (for review see Nishizawa and Laher, 2005). SOD1 overexpression has been shown to protect against vasospasm following SAH (Kamii et al., 1999), which may be due to inhibition of inducible NOS in the vascular wall (Saito et al., 2001), and similar results have been obtained with SOD mimetics (Aladag et al., 2003). Application of recombinant adenovirus encoding human SOD1 (gene transfer) to cerebral vessels has also been shown to reduce cerebral blood flow impairment in the acute stage of SAH (Shin et al., 2003).

Inflammatory responses with associated ROS production and alterations in antioxidant levels also contribute to the injury process following a hemorrhagic stroke. The role of SODs in inflammation is addressed in a subsequent section.

5.2 Ischemic Stroke

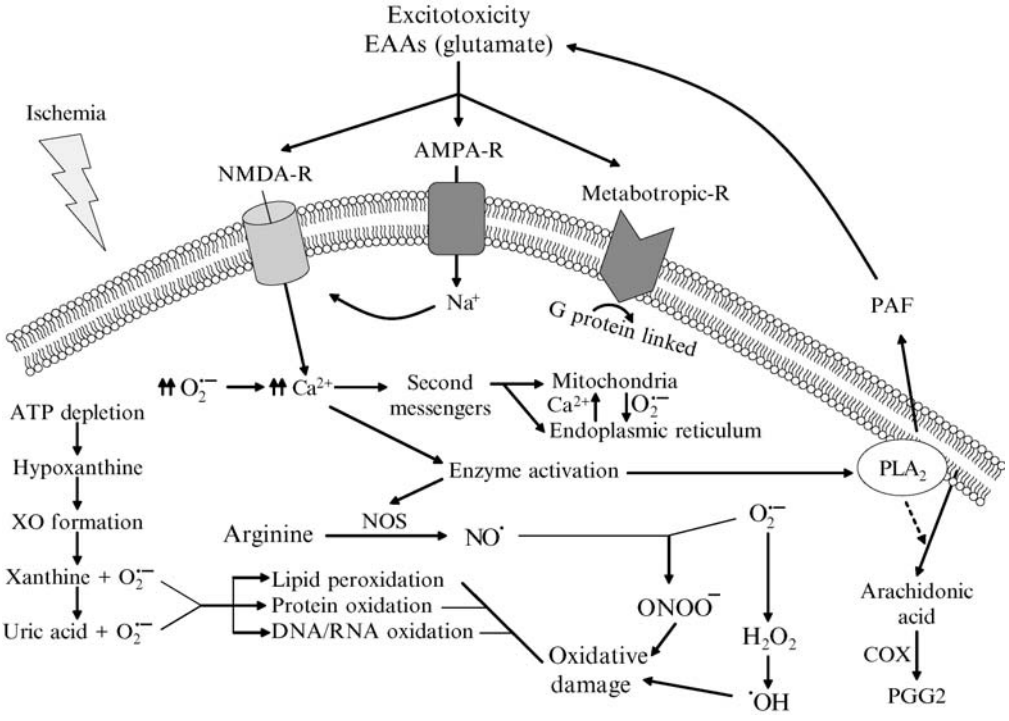
The pathophysiological mechanisms of ischemic brain injury involve time-dependent cascades of molecular events including rapid depletion of energy stores, generalized membrane depolarization, excitotoxicity, calcium overload, edema, mitochondrial dysfunction, free radical formation, immune activation, inhibition of protein synthesis, and alteration in gene expression (*Figure 8-3*; for review see Hayashi, 2004). Restoration of blood flow and recovery of the normal oxygen levels (reoxygenation) is essential to prevent the consequences of neuronal ischemia, but it can also result in reperfusion injury, a process that further damages brain cells, the ischemic arterial wall, and the microvasculature. SODs play a critical defensive role in the ischemic injury cascade, BBB damage, and the inflammatory processes that take place throughout the evolution of the infarct and well into the recovery phase of stroke.

5.2.1 The Acute (Within Hours) Stroke Phase

Expression of both intracellular SODs increases following an ischemic insult (Liu et al., 1993; Matsuyama et al., 1993) and the activity of these enzymes is differentially affected by the intensity of the ischemic

Figure 8-3

Possible mechanisms involved in oxidative damage. During an ischemic insult, ATP depletion from the lack of oxygen and glucose causes membrane depolarization (due to reduced activity of ATP-dependent ion pumps), followed by an influx of extracellular Ca^{2+} and a massive release of excitatory amino acids, such as glutamate. Glutamate activates both NMDA and non-NMDA-type ionotropic receptors, thereby further increasing the intracellular Ca^{2+} concentration and leading to the eventual activation of a variety of Ca^{2+}-stimulated enzymes (e.g., proteases, lipases, protein kinases, and nucleases). When phospholipase A2 is activated, a large amount of platelet-activating factor (PAF) is released into the intercellular space, thereby enhancing excitotoxicity. This also activates cyclooxygenase-2, which further synthesizes biologically active prostaglandins. Superoxide can peroxidate lipids and stimulate the release of glutamate, which in turn increases the production of free radicals, disturbs calcium-ATPase, and exacerbates brain edema formation. Altered function of the mitochondrial electron transport chain is a major source of superoxide, as is the hypoxanthine/xanthine oxidase system. During ischemia, ATP is catabolized to hypoxanthine, which accumulates in tissues. The ensuing influx of Ca^{2+} into the cell triggers the conversion of NAD^+-reducing xanthine dehydrogenase to xanthine oxidase (XO). Reperfusion provides the oxygen required for the reaction between hypoxanthine and XO, thus allowing the generation of superoxide (modified from Maier and Chan, 2002)



challenge. However, the exact time-line and relative contribution of SOD1 versus SOD2 in the early stages of ischemia and reperfusion are not completely understood. SOD activity has been detected in the ischemic brain within 30 min of the insult, but immunoreactivity has not been detected before 4 h following global ischemia (Kato et al., 1995) and 24 h following 1 h of focal ischemia (Liu et al., 1993). Of note, hyperglycemia has been shown to induce an early appearance (within 3 h of ischemic onset) of SOD2 following permanent focal cerebral ischemia, while increased SOD2 expression is not observed in normoglycemic animals until 24 h (Ste-Marie et al., 2001).

The fact that SOD1 is effective against transient but not permanent MCAO (Chan et al., 1993a; Yang et al., 1994; Kamii et al., 1996) suggests that this enzyme plays a protective role primarily during

reperfusion, although its constitutive expression would argue that SOD1 is a first line of defense against oxidative stress.

Superoxide generation peaks within the first 10–15 min of reperfusion and subsides in the subsequent 2 h (Peters et al., 1998). The first cells to come in contact with the high levels of oxygen and, thus ROS, are vascular cells. Excess superoxide formation results in alterations in vascular response to carbon dioxide and endothelium-dependent vasodilators such as acetylcholine, increased platelet aggregation, endothelial cell damage, and increased microvascular permeability. Recently, Brzezinska et al. (2005) have shown that superoxide directly affects the function of ion channels in vascular endothelial cells, an effect that can be reversed and prevented by SOD. In addition, SOD prevents abnormal vasoreactivity as well as increased permeability of the BBB. Another significant source of superoxide during the acute stroke comes from inflammatory cells. A variety of proinflammatory cytokines (e.g., TNFα, IL-1β, and IL-6), chemokines (e.g., monocyte chemoattractant protein 1 [MCP-1] and macrophage inflammatory protein 1α [MIP-1α]), adhesion molecules, and destructive enzymes (e.g., cyclooxygenase-2, NOS, and proteinases) are quickly upregulated in response to ischemia/reperfusion injury. Stimulated polymorphonuclear cells, such as neutrophils, which synthesize and release superoxide at a high rate, adhere to the vascular endothelium and begin to infiltrate infarcted tissue as early as 6 h after ischemia (Garcia et al., 1994; Maier et al., 1998). Similarly, within hours of the ischemic insult, cytokine-mediated activation of microglia results in superoxide production and subsequent release into the extracellular space (Hoffstein et al., 1985).

MCP-1and MIP-1α gene expression is upregulated as early as 3 h after transient MCAO, and SOD1 overexpression can suppress their mRNA expression, as well as that of TNFα and IL-6 (Nishi et al., 2005). Expression of MCP-1and MIP-1α is also increased after permanent MCAO but is delayed relative to reperfusion-related induction (Kim et al., 1995; Che et al., 2001). It is worth mentioning that all the proinflammatory cytokines and chemokines discussed earlier are controlled, to some degree, by NF-κB (Hallenbeck, 2002), and SOD1 can downregulate NF-κB expression (Huang et al., 2001).

5.2.2 The Subacute (Hours to Days) Stroke Phase

In patients with cerebral ischemia, serum SOD activity is significantly reduced within 24 h after onset of neurological deficits (and is inversely correlated with the size of infarction) but recovers to values not different from control subjects within 5 days after stroke (Spranger et al., 1997).

Cerebral edema is well under way at this stage of disease progression, and both intracellular SODs are effective at blunting edema formation during the reperfusion phase (Kokubo et al., 2002; Maier et al., 2006). The microglial reaction is also fully developed within the first 24 h of a focal ischemic insult, whereas reactions at sites remote to the injury (neocortex, thalamus, and both hippocampi) take about 5 days to become fully apparent (Gehrmann et al., 1995). At 2–3 days, there is also a massive invasion of monocytes (Garcia et al., 1994), and brain macrophages are still clearly visible at 3–7 days (Maier et al., 1998). Activation of the above-mentioned proinflammatory genes peaks at this stage, along with activation of transcription factors such as hypoxia-inducible factor 1, NF-κB, and interferon regulatory factor-1. The BBB is at high risk during this period, likely due to the considerable inflammatory response under way and, if the ischemic insult is of sufficient magnitude, hemorrhagic transformations can occur. Mediators of vascular damage include MMPs (e.g., MMP-9 and -2), and cytokines, which are activated by ROS. Interestingly, the significant increase in MMP levels observed during the subacute stroke phase comes not only from inflammatory cells but also from endothelial cells. Indeed, MMP-9-expressing vascular cells may play a more significant role in BBB disruption at this stage than neutrophils, although this idea is still controversial (Maier et al., 2004). It is important to note, however, that MMP expression also occurs early in stroke evolution (Heo et al., 1999). Irrespective of the cellular source of vascular injury mediators, SODs are protective during the subacute phase of stroke, and their absence or reduced activity levels can result in increased BBB leakage and hemorrhage rates (Gasche et al., 2001; Maier et al., 2006).

5.2.3 The Chronic (Days to Months) Stroke Phase

Inflammatory cells are still visible weeks after stroke onset, but their role is confined primarily to removal of dead and injured tissue. Recovery processes are also under way, including angiogenesis as well as limited neurogenesis. SOD2 immunoreactivity is still visible 2 months after a transient focal stroke but is restricted mostly to the infarct border (Maier et al., 2002). Interestingly, at this time point, reactive astrocytes are evident in the rim of the lesion due to gliosis and scar formation. Recently, Yanpallewar et al. (2005) have shown that reactive changes in brain histology, like gliosis, are attenuated by treatment with *Azadirachta indica*, a plant that has been reported to possess antioxidant and antiinflammatory properties.

Until recently, the primary goal of acute stroke research has been to develop agents that can reduce infarct volume, thereby limiting neurological deficits. Now, cell transplantation and enhanced neurogenesis are key therapeutic strategies being investigated for stroke treatment (for review see Zhang et al., 2005). The role of SODs in these processes remains to be determined, although there are emerging data suggesting that neural precursor cells may be uniquely predisposed to redox regulation and changes in antioxidant levels. For example, Limoli et al. (2004) have recently shown that ROS levels, which depend critically on changes in cell density, are significantly higher in neural precursor cells compared with other cell lines. Furthermore, at high neural precursor cell densities, intracellular ROS and irradiation-induced oxidative damage are reduced with a concomitant increase in SOD2 expression.

6 SOD-Based Therapeutic Strategies

The pharmacodynamic and pharmacokinetic properties of the SOD isoenzymes present practical obstacles to using the naturally occurring forms of SOD as therapeutic agents. The short half-life of native SOD1, its high renal clearance, and its failure to cross the BBB and be absorbed intracellularly have likely contributed to the mixed results obtained in therapeutic trials (Chan et al., 1993b). Similar obstacles have been encountered with SOD2. The positively charged region in the hydrophilic C terminus of each SOD3 subunit promotes binding of this enzyme to cell surfaces. Thus, exogenously administered SOD3 may not reach its intended target.

To circumvent these problems, researchers have made various attempts at modifying the proteins, such as conjugating the inert polymer polyethylene glycol (PEG) to amino groups on SOD. These modifications have resulted in increased half-life, BBB permeability, and cellular uptake and have improved their effectiveness against ischemic injury (Chan et al., 1987; Imaizumi et al., 1990). More recently, therapeutic approaches for using SOD have included enhancing native SOD activity and/or gene expression, using more sophisticated SOD mimetics, and supplementary enzyme therapy (Johnson and Giulivi, 2005). Augmentation of native SOD activity can be exemplified by the dihydropyridine calcium antagonist amlodipine. This antihypertensive agent can slow the progression of atherosclerosis (Pitt et al., 2000), inhibit cardiovascular events (Major outcomes in high-risk hypertensive patients randomized to angiotensin-converting enzyme inhibitor or calcium channel blocker versus diuretic, 2002), and has been shown to upregulate SOD1 activity in heart tissue (Umemoto et al., 2004). Nifedipine, another calcium antagonist, has also been shown to indirectly upregulate endothelial SOD expression by stimulating vascular endothelial growth factor production from adjacent vascular smooth muscle cells (Fukuo et al., 2002) and to induce upregulation of SOD2 in VSMCs via NO derived from endothelial cells (Fukuo et al., 2003). Natural extracts that enhance cellular antioxidant abilities are also under investigation (Ng et al., 2005).

The use of porphyrin-based or salen-MnSOD mimetics is another therapeutic approach being studied for pathophysiological conditions associated with superoxide anion overproduction (for review see Salvemini et al., 2002). SOD mimetic drugs have been shown effective in controlling hypertension (Schnackenberg et al., 1998; Hahn et al., 1999; Vaziri et al., 2001), reducing hypoxic-ischemic brain damage (Baker et al., 1998; Mollace et al., 2003; Shimizu et al., 2003; Mehta et al., 2004; Lee et al., 2005), reversing endothelial cell dysfunction (Nassar et al., 2002; Jiang et al., 2003), and attenuating cerebral vasoconstriction following SAH (Aladag et al., 2003). Although there have been many advances in the development of

SOD mimetics, low plasma membrane permeability continues to be an obstacle for their clinical use. Thus, much effort is being directed toward improving the bioavailability and lipophilicity of these compounds (Ferrer-Sueta et al., 2003; Trostchansky et al., 2003; Batinic-Haberle et al., 2004; Okado-Matsumoto et al., 2004). In addition to concerns with SOD mimetic biological properties, there are also questions regarding their specificity and even mode of action. Recently, Tauskela et al. (2005) found that some Mn-based metalloporphyrins, acting at the plasma membrane, protect neurons against oxygen/glucose deprivation by suppressing postsynaptic NMDA receptor-mediated calcium rises and not by acting as intracellular catalytic antioxidants. Therefore, they caution that future work on SOD mimetics should consider whether oxidative- or calcium-based stress is to be the target of neuroprotection, and suggest that synthetic compounds targeting both oxidative stress and antioxidant-independent components of cellular injury may have greater therapeutic potential.

Enhanced therapeutic efficacy of SOD mimetics is also being attempted by targeting these antioxidants to cellular sites of oxidant generation, such as the mitochondria, although one such SOD mimetic has failed to exhibit higher efficacy than the mitochondrial nontargeted analog (Dessolin et al., 2002). Targeting lipid membranes and lipoproteins is another strategy. For example, the Mn(II) and Cu(II) complexes of the antibiotic ionophore monensin have been shown to exhibit considerable superoxide-scavenging activities and may represent a novel class of lipophilic catalytic antioxidants for the protection of lipid structures (Fisher et al., 2005). Another therapeutic approach involves the construction of chimeric proteins such as the SOD2/3 generated by Gao et al. (2003). This chimeric fusion SOD is composed of human SOD2 primary structure plus the 26 amino acid C terminus of human SOD3 and appears to have therapeutic anti-inflammatory properties that are greatly superior to the three naturally occurring forms of the human enzyme. Recently, Bonder et al. (2004) have shown that SOD2/3 administration prevents ischemia/reperfusion-induced neutrophil-endothelial cell interactions and microvascular dysfunction.

Advances in protein transduction technology have allowed researchers to generate a Tat-SOD fusion protein that is efficiently transduced into mammalian cells. Transduced Tat-SOD has been shown to be enzymatically and biologically active in cells and tissues and to protect against neuronal cell death in vitro as well as following transient forebrain ischemia (Kim et al., 2005).

Although these advances have brought us closer to using SOD for stroke treatment, the need to maintain an oxidant–antioxidant balance and the ability to target the affected cells/tissues at the appropriate stage of disease progression remain the biggest obstacle to using SOD for stroke treatment.

7 Summary

Understanding the exact mechanisms that predispose the brain to ischemia-induced oxidative injury, the pathophysiology involved in lesion growth, and the recovery mechanisms following a stroke is critical for the identification of appropriate therapeutic targets and thus the development of treatment strategies. Over the past few years, our knowledge regarding the role of ROS and SOD isoenzymes in stroke etiology and evolution has increased substantially, but there is still a great deal more to be learned. In addition to their role in cell death mechanisms, it is now clear that ROS and SODs are also important in modulating the expression of survival signaling pathways. Some of these survival mechanisms may extend beyond the acute and subacute phases of stroke, but the role of SODs in recovery processes such as angiogenesis and neurogenesis as well as in brain plasticity and reorganization following a cerebrovascular accident remains to be elucidated.

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9 The Role of Glia in Excitotoxicity and Stroke

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Abstract: Neurons are highly integrated both anatomically and metabolically with glial cells, and thus glial cells have a major influence on neuronal survival in ischemia and excitotoxicity. Of the three types of glia in the central nervous system—astrocytes, oligodendrocytes, and microglia—the role of astrocytes in excitotoxicity and ischemia has been best characterized. Under different settings, astrocytes can both limit or contribute to excitotoxic neuronal death. Astrocytes also influence oxidative neuronal injury and contribute to neuronal demise through secretion of nitric oxide and cytokines. Microglia, the resident macrophages of the CNS, can also have both deleterious and salutary effects on neuronal survival. Activated microglia can kill neurons, but on the other hand normal microglial function is probably required for brain remodeling after injury. Interactions between microglia and astrocytes engender an additional layer of complexity to these post-ischemic processes.

List of Abbreviations: AA, arachidonic acid; Ala, alanine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPA/KAR, AMPA/kainate receptor; APP, amyloid precursor protein; ATP, adenosine triphosphate; BCAA, branched amino acid; BDNF, brain-derived neurotrophic factor ; bFGF, basic fibroblast growth factor; CDNF, ciliary neurotrophic factor; COX-2, cyclooxygenase; cPLA2, cytoplasmic phospholipase A; DNA, deoxyribonucleic acid; iNOS, inducible nitric oxide synthase; GDNF, glial cell-derived neurotrophic factor; Glu, glutamate; Gln, glutamine; CNS, central nervous system; EAAT, excitatory amino acid transporter; GABA, gamma-aminobutyric acid; GLAST, glial glutamate and aspartate transporter; GLT-1, glial glutamate transporter; GlyT, glycine transporter; GM-CSF, granulocyte-magrophage colony-stimulating factor; GSH, glutathione; HIF-1, hypoxia-inducible factor-1; HO-1, heme oxygenase -1; ICAM-1, intracellular adhesion molecule-1; ICE, interleukin-1 converting enzyme; IGF-I, insulin growth factor I; IL, interleukin; JAK-2, janus tyrosine kinase-2; αKG, α-ketoglutarate; KIC, α-ketoisocaproate; MAPK, mitogen-activated protein kinase; M-CSF, macrophage colony-stimulating factor; MHC, major histocompatibility complex; MIP-1, macrophage inflammatory protein-1; MMP, matrix metalloproteases; NF-κB, nuclear factor-kappaB; NGF, nerve growth factor; NMDA, N-methyl-D-aspartate; NT-3, neurotrophin-3; PA, plasminogen activator; PARP-1, poly(ADP-ribose) polymerase-1; PDGF, platelet derived growth factor; PG, prostaglandin; PKC, protein kinase C; PYK2, proline-rich protein tyrosine kinase; Pyr, puryvate; RANTES, regulation on activation normal T-cell expressed and secreted; SAT, sodium-coupled amino acid transporter; TCA, tricarboxylic acid cycle; TGF, transforming growth factor; TIMP, tissue-specific inhibitor of metalloproteinase; TNF, tumor necrosis factor; t-PA, tissue-specific plasminogen activator; u-PA, urokinase-plasminogen activator; VEGF, vascular endothelial growth factor; VSOAC, volume-sensitive organic anion channel

1 Introduction

Excitotoxic neuronal death is triggered by the sustained activation of glutamate receptors. During brain ischemia, glutamate receptor activation results from massive, sustained elevation in extracellular glutamate concentrations. Neurons are highly integrated both anatomically and metabolically with glial cells, and accordingly the glia have a major impact on neuronal survival in ischemia and excitotoxicity. Of the three types of glia in the central nervous system (CNS)—astrocytes, oligodendrocytes, and microglia—the role of astrocytes in excitotoxicity and ischemia has been best characterized. Under different settings, astrocytes can both limit or contribute to excitotoxic neuronal death. Microglia, the resident macrophages of the CNS, can also contribute to injury resulting from excitotoxicity and stroke, particularly in the postischemic period. The mechanisms by which these glial cell types influence ischemic neuronal survival will be discussed in turn.

2 Excitotoxicity in Stroke

Reductions in blood flow to less than roughly 20% of normal for more than a few minutes leads to a massive increase in extracellular glutamate (Benveniste et al., 1984). The extent and duration of this increase depend

on the severity and duration of the ischemic insult. With ischemia of 10-min duration, there is a transient rise of glutamate followed by a second higher peak of increase during reperfusion (Caragine et al., 1998). Studies with glutamate receptor antagonists have conclusively shown the importance of this cell death pathway in animal models of stroke (Simon et al., 1984; Meldrum et al., 1987; Choi, 1988; Ozyurt et al., 1988; Swan and Meldrum, 1990; Le Peillet et al., 1992). As might be expected, however, the efficacy of these agents is much decreased when administered at time points after glutamate elevations have occurred.

2.1 Mechanisms of Glutamate-Induced Cell Death

Glutamate binding to neuronal *N*-methyl-D-aspartate (NMDA) type glutamate receptors triggers an influx of Na^+, K^+, and Ca^{2+}, which, if sustained, can lead to neuronal death. Key events in the excitotoxic cell death pathway are dysregulation of intracellular calcium homeostasis and the generation of reactive oxygen species. Reactive oxygen species are formed especially during reperfusion after ischemia and include superoxide, nitric oxide, peroxynitrite, and hydrogen peroxide. These agents can damage many critical cell components, but in particular they damage DNA. DNA damage, in turn, can lead to either apoptotic or necrotic cell death.

Glutamate is not the only factor determining NMDA receptor activity. NMDA receptor activity is positively modulated by glycine and D-serine (White et al., 2000) and neuronal membrane depolarization (Novelli et al., 1988; Greene and Greenamyre, 1996). Conversely, NMDA receptor activity is attenuated by coactivation of neuronal GABA and purinergic receptors (Muir et al., 1996; Ortinau et al., 2003).

3 Astrocyte Modulation of Glutamate Neurotoxicity

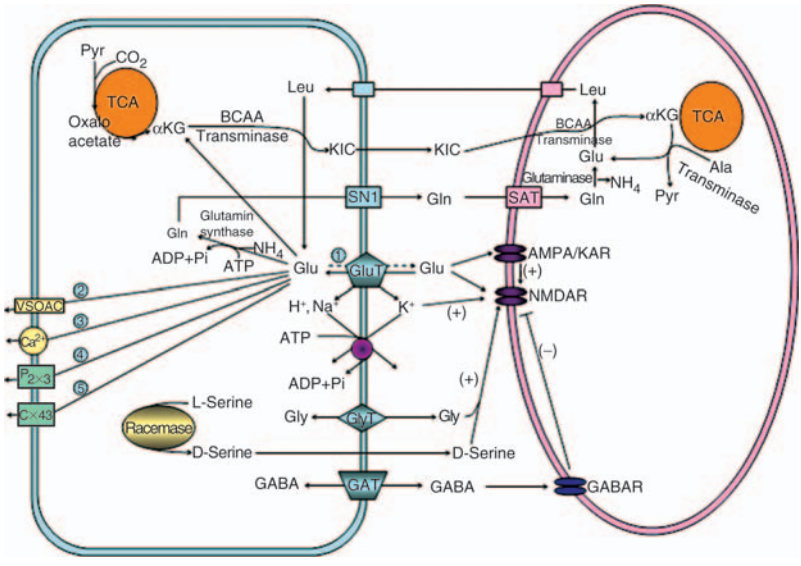
3.1 Astrocyte Glutamate Uptake

Glutamate uptake is the most well-characterized mechanism by which astrocytes influence neuronal survival during ischemia, and this topic has been the subject of recent thorough reviews (Schousboe et al., 1997; Robinson, 1998; Vandenberg, 1998; Anderson and Swanson, 2000; Danbolt, 2001). Clearance of glutamate from the extracellular space is accomplished primarily by Na^+-dependent transporters localized on astrocytes (*Figure 9-1*). There are two main subtypes of glutamate transporters expressed by astrocytes. These were originally cloned from the rat brain and termed GLAST; glial glutamate and aspartate transporter and GLT-1; glial glutamate transporter. Human homologs of these transporters are termed EAAT1 and EAAT2; excitatory amino and transporter 1 and 2 respectively. In cortical cultures, neuronal vulnerability to glutamate is 100-fold greater in astrocyte-poor cultures than in cultures with abundant astrocytes (Rosenberg and Aizenman, 1989), suggesting that uptake by astrocytes can be a limiting factor in glutamate neurotoxicity. The dominant role of astrocyte transporters is further supported by studies using genetic downregulation of transporter subtypes. Antisense "knockdown" of GLAST or GLT-1, but not the neuronal subtype EAAC1, produces excitotoxic neurodegeneration and increased susceptibility to seizures and injury (Rothstein et al., 1996; Tanaka et al., 1997; Watase et al., 1998). Likewise, genetic downregulation of the glial glutamate transporter GLT-1, but not the neuronal glutamate transporter EAAC1, was shown to exacerbate ischemic neuronal damage in the rat brain (Rao et al., 2001), thereby establishing the importance of astrocyte uptake for neuronal survival during ischemia.

Glutamate uptake is energetically costly, requiring roughly 1 ATP per glutamate molecule transported. Given the high energetic cost of glutamate uptake, it is not surprising that complete ischemia leads to abrupt cessation of glutamate uptake and a massive increase in extracellular glutamate concentrations (Benveniste et al., 1984; Choi, 1988; Swanson et al., 1994). During incomplete ischemia, however, continued supply of glucose can fuel continued ATP production by glycolysis, even in the absence of oxygen. Glycolytic ATP production appears sufficient to fuel glutamate uptake in astrocytes (Hakim, 1987; Swanson, 1992; Swanson et al., 1994). However, acidosis is a significant factor determining the ability of astrocytes to maintain ATP levels in the absence of oxygen delivery, and the ability of astrocytes to maintain glutamate uptake during

Figure 9-1

Astrocyte modulation of glutamate excitotoxicity. Neuronal glutamate (Glu) is formed chiefly from astrocyte-derived glutamine (Gln). Gln is formed in astrocytes from glutamate by glutamine synthase and then released from astrocytes via system N transporters (SN1). Gln is taken up by neurons through Na^+-coupled amino acid transporter (SAT). In neurons, Gln is converted back to Glu by glutaminase. Neuronal Glu is also formed from astrocyte-derived α-ketoisocaproate (KIC). KIC is formed in astrocytes by the action of branched amino acid (BCAA) transaminase, whereby leucine (Leu) and α-ketoglutarate (αKG) are converted to KIC and glutamate. KIC is used by neurons for the reverse reaction as an anaplerotic reaction to replenish tricarboxylic acid (TCA) cycle intermediates that are expended for amino acid neurotransmitter release. Astrocyte uptake of glutamate (Glu) by glutamate transporters (GluT) limits glutamate access to neuronal NMDA receptors (NMDAR). The transporters link glutamate transport to the cotransport of 3 Na^+ and 1 H^+ and the counter-transport of 1 K^+ ion. Uptake is thus metabolically coupled to the astrocyte plasma membrane Na^+/K^+-ATPase. NMDA receptor activity is positively modulated by D-serine produced by L-serine racemase in astrocytes, and by glycine, which is actively transported by astrocytes (GlyT). Glutamate binding to NMDA receptor is also positively modulated by neuronal membrane depolarization induced by glutamate activation of AMPA/kainite receptors (AMPA/KAR) and by increased extracellular K^+. Astrocyte uptake of glycine, GABA, and glutamate (1) can also proceed in the reversal direction during astrocyte energy failure. Astrocytes can also release glutamate through volume-sensitive organic anion channels (VSOACs) (2), by Ca^{2+}-dependent, vesicular release in response to various signaling molecules (3), by ATP-gated P2x7 receptor channels (4), and by unpaired connexin-43 hemichannels (Cx-43) (5). Puryvate (Pyr); alanine (Ala); phosphate (Pi)



hypoxia is markedly impaired by acidosis over the pH range resulting from ischemia (Swanson et al., 1995). Oxidative stress-induced activation of poly(ADP-ribose) polymerase-1 (PARP-1) in astrocytes may additionally compromise energy supply, in part, by limiting glycolysis (Ying et al., 2002). PARP-1 activation has been shown to impair astrocyte glutamate uptake in vitro (Swanson et al., 2002), suggesting that this mechanism may also contribute to impaired glutamate uptake during and after ischemia.

Glutamate uptake capacity is dynamically regulated. Astrocytes in culture can rapidly increase glutamate transport capacity in response to elevated extracellular glutamate concentrations by a process mediated by glutamate-induced translocation of glutamate transporters from intracellular space to plasma membranes (Duan et al., 1999; Robinson, 2002); however, this process is unlikely to be an important determinant of

uptake capacity during ischemia in which energy production is the rate-limiting factor. On the other hand, several factors can downregulate astrocyte glutamate transport, and these may further compromise glutamate uptake under ischemic conditions. Reactive oxygen species, particularly peroxynitrite, can reduce glutamate transporter function (Pogun et al., 1994; Volterra et al., 1994; Trotti et al., 1996; Trotti et al., 1998; Chen et al., 2000), possibly through interactions with specific protein sulfhydryl groups. Arachidonic acid (Trotti et al., 1995; Zerangue et al., 1995), endothelin-1 (Leonova et al., 2001), and zinc (Vandenberg et al., 1998) are also released during ischemia and can downregulate or inhibit astrocyte glutamate uptake.

3.2 Astrocyte Glutamate Release

Glutamate transporters, like other transporters, are capable of moving substrates in both inward and outward directions (Szatkowski et al., 1990). In the setting of ATP depletion, as occurs during severe ischemia, the ATP-supported membrane gradients collapse. This results not only in cessation of uptake but also efflux of glutamate via uptake reversal. ATP depletion has been shown to cause uptake reversal in primary astrocyte cultures (Gemba et al., 1994; Longuemare and Swanson, 1995), retina (Zeevalk et al., 1998), spinal cord and brain slice preparations (Li et al., 1999), and in the intact brain (Seki et al., 1999). These studies suggest that the rise in extracellular glutamate in the brain that occurs during energy failure may be due not only to failure of astrocyte glutamate uptake but also to glutamate efflux from astrocytes via reversal of the astrocyte glutamate transporters. However, there is controversy as to whether it is astrocytes or neurons that are the primary source of glutamate released by uptake reversal during ischemia. Studies in brain slice preparations suggest that glutamate efflux from neurons may be quantitatively more important, particularly at early time points (Rossi et al., 2000; Danbolt, 2001; Hamann et al., 2002). On the other hand, in vivo microdialysis studies have shown that dihydrokainate, which preferentially blocks the astrocyte transporter GLT-1, significantly attenuates the rise of extracellular glutamate that occurs during brain ischemia (Seki et al., 1999; Dawson et al., 2000).

Glutamate efflux from astrocytes can also occur by several other routes. Astrocyte swelling induced by elevated extracellular potassium or other factors can induce glutamate efflux via volume-sensitive anion channels (Kimelberg et al., 1990; Rutledge et al., 1998; Longuemare et al., 1999). Astrocytes can also release glutamate in response to bradykinin (Jeftinija et al., 1996) and prostaglandin E2 (Bezzi et al., 1998) in a manner that is sensitive to botulinum B neurotoxin. Glutamate release from astrocytes can be induced by extracellular ATP activation of astrocyte $P2X_7$ receptor channels (Duan et al., 2003). A recent study also reports glutamate release through unpaired connexin-43 hemichannels (Ye et al., 2003). However, the significance of glutamate release during ischemia by routes other than uptake reversal and volume-sensitive anion channels remains to be established.

3.3 Astrocyte Support of Neuronal Glutamate Release

Glutamine released by astrocytes is the major precursor for neurotransmitter glutamate synthesis. Glutamate released at neuronal synapses is taken up by surrounding astrocytes (McLennan, 1976; Rothstein et al., 1996) and converted by glutamine synthetase to glutamine. Glutamine is in turn released from astrocytes for uptake by neurons (Bradford et al., 1978; Waniewski and Martin, 1986; Broer and Brookes, 2001; Chaudhry et al., 2002), where it is metabolized back to glutamate and packaged into synaptic vesicles. Pharmacological inhibition of glutamine synthetase reduces brain glutamine levels, reduces K^+-evoked glutamate release (Paulsen and Fonnum, 1989), and has been reported to reduce infarct size in a focal ischemia model of stroke (Swanson et al., 1990). The cycle of glutamate carbon through neurons and astrocytes is supplemented by a similar flux of branched-chain amino acids in which transamination of leucine and α-ketoglutarate in astrocytes produces glutamate and α-ketoisocaproic acid (Yudkoff et al., 1996). α-Ketoisocaproic acid is released to the extracellular fluid and may be taken up by neurons as a substrate for the reverse reaction.

3.4 Astrocyte Regulation of Extracellular GABA, Glycine, and D-Serine

GABA is an inhibitory neurotransmitter that can attenuate the effects of glutamate receptor stimulation (Muir et al., 1996). GABA transporters are expressed by astrocytes, and pharmacological inhibitors that target astrocyte GABA uptake exhibit anticonvulsant activity (Schousboe, 2000). However, GABA transporters are also widely expressed by neurons (Palacin et al., 1998; Gadea and Lopez-Colome, 2001b), and the specific contribution of astrocyte GABA uptake under normal or pathological conditions remains uncertain (Schousboe, 2000).

Glycine and D-serine act as positive modulators of signal transduction at NMDA-type glutamate receptors (Wroblewski et al., 1989), and these compounds can significantly influence glutamate excitotoxicity during ischemia (Foster et al., 1990). Astrocytes can both release and take up glycine from the extracellular space through the GlyT1 glycine transporter (Gadea and Lopez-Colome, 2001a; Supplisson and Roux, 2002). Interestingly, the kinetic properties of GlyT1 differ from those of the dominant neuronal transporter, GlyT2 (Supplisson and Roux, 2002). Glycine uptake by GlyT2 is coupled to the cotransport of 3 Na^+ and 1 Cl^-, whereas uptake by astrocyte GlyT1 is coupled to 2 Na^+ and 1 Cl^-. This allows neurons to maintain a higher intracellular glycine concentration, a factor that may facilitate neuronal loading of glycine into synaptic vesicles. Higher neuronal intracellular glycine concentrations are also favored by a kinetic restraint on reverse operation of the GlyT2 transporters. By contrast, astrocyte GlyT1 transporters readily function in "reverse" mode, and because these transporters operate near equilibrium, one function of astrocyte GlyT1 transporters may be to allow efflux of glycine into the extracellular space. D-Serine is generated from L-serine by racemase that is exclusively expressed in the protoplasmic astrocytes that typically ensheath synapses (Wolosker et al., 1999; Snyder and Ferris, 2000). Activation of glutamate receptors on astrocytes leads to activation of L-serine racemase and astrocyte release of D-serine. Because D-serine, like glycine, is a positive modulator of neuronal NMDA receptors, this process may contribute to excitotoxic neuronal death (Foster et al., 1990; Snyder and Ferris, 2000).

4 Astrocyte Influences on Downstream Events in Excitotoxic Cell Death

4.1 Astrocyte Influences on Oxidative Neuronal Injury

Reactive oxygen species are generated by several mechanisms during ischemia and ischemia-reperfusion, and the resulting oxidative stress constitutes a major mechanism of ischemic neuronal injury (Chan, 2001). Glutathione (GSH) is the principal antioxidant in brain (Dringen, 2000), and brains depleted of glutathione are sensitized to ischemic injury (Mizui et al., 1992). Evidence suggests that astrocytes contain greater concentrations of glutathione and enzymes involved in glutathione metabolism than neurons (Slivka et al., 1987; Yudkoff et al., 1990; Makar et al., 1994; Wilson, 1997). Similarly, glucose flux through the pentose phosphate pathway in cultured astrocytes is twice that of cultured neurons and increases three times as much as in neurons during H_2O_2 exposure (Ben-Yoseph et al., 1996). These factors suggest that astrocytes are more capable of scavenging reactive oxygen species than neurons and suggest that oxidant-scavenging mechanisms in astrocytes may function to support neuronal survival. In support of this idea, neurons cultured in the presence of astrocytes are more resistant than neurons cultured alone to injury induced by nitric oxide, hydrogen peroxide, or superoxide (Desagher et al., 1996; Lucius and Sievers, 1996; Tanaka et al., 1999b; Xu et al., 1999). Moreover, astrocytes depleted of glutathione show reduced ability to protect neurons from oxidant injury (Drukarch et al., 1997; McNaught and Jenner, 1999; Chen et al., 2001). The effects of astrocyte glutathione on neuronal resistance to oxidative stress may be mediated, in part, by maintaining neuronal glutathione levels. Astrocytes contribute to neuronal glutathione content by an indirect route: glutathione is released by astrocytes and cleaved to the dipeptide CysGly (Dringen et al., 1999), which in turn is cleaved to free cysteine for uptake into neurons as a substrate for neuronal GSH synthesis (Dringen et al., 2001).

Like glutathione, ascorbate is an important antioxidant that is present in the brain at millimolar concentrations (Rice, 2000). Ascorbate can directly react with oxidants and can also serve as a cofactor for reducing

(recycling) oxidized glutathione and α-tocopherol. Evidence suggests an ascorbate cycle between neurons and astrocytes. Neurons release oxidized ascorbate (dehydroascorbate) for uptake by astrocytes, which then convert it to ascorbate and, in turn, release ascorbate for neuronal uptake (Siushansian and Wilson, 1995; Siushansian et al., 1997; Wilson, 1997; Daskalopoulos et al., 2002). Dehydroascorbate passes across the blood–brain barrier readily and is converted to ascorbate, presumably in astrocytes. Treatment with dehydroascorbate has been shown to attenuate ischemic brain injury, suggesting that this cycle is a significant aspect of astrocyte–neuron interaction during ischemia (Huang et al., 2001).

4.2 Erythropoietin Released by Astrocytes Blocks Ischemic Neuronal Death

The glycoprotein hormone erythropoietin is produced in the CNS as well as in the periphery and has substantial effects on neuronal survival after ischemia (Buemi et al., 2003). Evidence suggests that erythropoietin production in the CNS is localized primarily to astrocytes (Masuda et al., 1994; Nagai et al., 2001; Ruscher et al., 2002), although some studies also report expression by neurons (Bernaudin et al., 2000). Erythropoietin activation of neuronal erythropoietin receptors blocks cell death pathways triggered by excitotoxicity and by combined oxygen/glucose deprivation in neuronal cultures (Morishita et al., 1997; Sakanaka et al., 1998; Ruscher et al., 2002; Wen et al., 2002) and reduces neuronal death after ischemia in vivo (Sakanaka et al., 1998; Siren et al., 2001; Wen et al., 2002). Neurons stimulated by erythropoietin show inhibition of caspase-9, increased expression of Bcl-2 family antiapoptotic factors, increased antioxidant capacity, and several other changes that may favor survival during and after ischemia (Calapai et al., 2000; Kawakami et al., 2001; Ruscher et al., 2002; Wen et al., 2002). These effects are mediated by activation of Janus tyrosine kinase-2 (JAK-2) pathway, which in turn activates Akt (protein kinase B) and the transcription factors nuclear factor-kappa B (NF-κB) and STAT5 (Digicaylioglu and Lipton, 2001; Kawakami et al., 2001; Siren et al., 2001; Ruscher et al., 2002). Astrocyte production of erythropoietin is increased after ischemic stress as a result of hypoxia-inducible factor-1 (HIF-1) activation, and postischemic erythropoietin production has been identified as a mediator of ischemic tolerance induced by sublethal cerebral ischemia (Ruscher et al., 2002; Prass et al., 2003).

5 The Astrocyte Inflammatory Response

The reaction of astrocytes to ischemia and other brain insults is similar to the inflammatory response of peripheral tissues. Within a few hours of virtually any type of brain injury, surviving astrocytes in the affected region begin to exhibit hypertrophy and proliferation (Ridet et al., 1997). This response, termed reactive astrogliosis, is fortified by migration of microglia and macrophages to the damaged area. Reactive astrocytes increase the expression of their structural proteins, GFAP and vimentin (Eng et al., 2000), as well as many other proteins. Cu/Zn superoxide dismutase, glutathione peroxidase, and metallothionein are increased in reactive astrocytes after ischemia (Liu et al., 1993; Takizawa et al., 1994; Neal et al., 1996; Campagne et al., 2000), indicating an enhanced capacity to neutralize reactive oxygen species. Similarly, astrocytes express the inducible form of heme oxygenase-1 (HO-1) in response to ischemia and other brain insults (Geddes et al., 1996; Takeda et al., 1996). HO-1 is the first step of heme metabolism and may be important in preventing heme iron participation in metal-catalyzed free radical production, particularly after conditions such as trauma or hemorrhagic stroke that liberate hemoglobin into the brain parenchyma. Other aspects of the astrocyte inflammatory response, such as nitric oxide and matrix metalloproteinase (MMP) expression, may be adaptive in settings such as infection but contribute to delayed neuronal death in settings such as stroke. (R)-(-)-2-propyloctanoic acid, an agent that suppresses the astrocyte inflammatory response, has been shown to reduce infarct size expansion when administered after cerebral ischemia (Matsui et al., 2002; Tateishi et al., 2002), although the detailed mechanism of this effect is not yet established.

5.1 Astrocyte Expression of iNOS and Cytokines

Expression of inducible nitric oxide synthase (iNOS) is a salient aspect of the astrocyte inflammatory response (Endoh et al., 1993). Astrocyte iNOS expression is detectable within a few hours of ischemia and is maximal within 2–3 days (Iadecola et al., 1995; Nakashima et al., 1995). Nitric oxide is a reactive oxygen species that can contribute to neuronal cell death by potentiating glutamate excitotoxicity (Hewett et al., 1994) and by several other mechanisms (Dawson and Dawson, 1998). Mice that are genetically deficient in iNOS exhibited smaller infarct size than control, wild-type mice (Iadecola et al., 1997), although this effect may also be due to reduced iNOS expression in microglia and invading macrophages.

Astrocytes stimulated by ischemic injury also produce many cytokines, including tumor necrosis factors (TNF-α, -β), interleukins (IL-1, -6, -10), and interferons (IFN-α, -β) (Feuerstein et al., 1998; Dong and Benveniste, 2001). The net effect of individual cytokines can be difficult to establish because the effects of many cytokines are strongly influenced by one another and because most cytokines have pleiotropic and cell-type specific effects. For example, IL-6 and TNF-α have been shown to promote demyelination, thrombosis, leukocyte infiltration, and blood–brain barrier disruption (Feuerstein et al., 1998; Dong and Benveniste, 2001). On the other hand, IL-6 has been shown to protect against ischemic and excitotoxic injury (Maeda et al., 1994; Ali et al., 2000), and hippocampal neurons treated with TNF-α are less vulnerable to substrate deprivation and excitotoxicity (Cheng et al., 1994). The specific contribution of astrocyte cytokine release to these processes in vivo remains to be established.

A recently identified regulator of inflammatory processes is PARP-1. PARP-1 is known to function in DNA repair (Ha and Snyder, 2000) but also functions as a coactivator of NF-κB (Kameoka et al., 2000; Chiarugi and Moskowitz, 2003), a transcription factor that plays an important role in the expression of inflammatory mediators. The formation of nuclear PARP-1/NF-κB complex has been shown to enhance DNA binding of NF-κB and transcription of NF-κB-regulated genes. Whether the enzymatic activity of PARP-1 is essential in this process remains unsettled (Chang and Alvarez-Gonzalez, 2001; Ullrich et al., 2001; Chiarugi and Moskowitz, 2003). Downregulation of PARP-1 causes a large reduction in brain infarct size after ischemia (Eliasson et al., 1997). It is likely that this large effect is due, in part, to an attenuated astrocyte inflammatory response, but there has been little study on this point.

5.2 Astrocyte Release of Matrix Metalloproteases

Cytokine and other proinflammatory stimuli of astrocytes in culture have been shown to induce activation of MMPs. MMPs are endopeptidases that are able to cleave protein components of extracellular matrix and are thus associated with tissue remodeling during developmental and pathological processes (Gottschall and Deb, 1996; Lee et al., 2003). Cytokines also modulate components of the plasminogen activator system, urokinase type plasminogen activator (u-PA) and tissue-specific plasminogen activator (t-PA), which are released by astrocytes and other cell types. These agents promote formation of (active) plasmin from (inactive) plasminogen and thus mediate cleavage of pro-MMPs into their active form (Faber-Elman et al., 1995; Tang et al., 2000). MMPs have been shown to not only cleave cytokines like TNF-α and IL-1β from their immature pro forms into their active mature forms (Gearing et al., 1995; Schonbeck et al., 1998; English et al., 2000) but also counterbalance the IL-1β activity by degrading the mature cytokine (Ito et al., 1996). Astrocytes release both MMP-2 and -9 (Rosenberg et al., 2001). Cerebral ischemia has been shown to increase MMP-2 activity in endfeet of rat brain astrocytes at time points (3 h and 5–21 days) relevant to changes in blood–brain barrier permeability, suggesting that astrocyte MMP-2 contributes both to blood–brain barrier opening and to later repair processes (Rosenberg et al., 2001). Astrocytes also express a tissue-specific inhibitor of metalloproteinase-1 (TIMP-1) and inhibitors of plasminogen activators (Tang et al., 2000).

6 Astrocyte Trophic Factor Release

Astrocytes release a variety of trophic factors under normal conditions, and these are likely to influence neuronal survival and plasticity after brain injury. These trophic factors include nerve growth factor (NGF),

basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and others (Ridet et al., 1997). Reactive astrocytes increase the expression of several of these, notably NGF, bFGF, BDNF, and neuregulins, which can stimulate neurite outgrowth (Schwartz and Nishiyama, 1994; Strauss et al., 1994; Mocchetti and Wrathall, 1995; Tokita et al., 2001). Reactive astrocytes also overexpress neuropilin-1 and vascular endothelial growth factor (VEGF), which act in concert to promote angiogenesis after cerebral ischemia (Zhang and Chopp, 2002). Although these findings suggest that astrocytes may play an important role in functional recovery after stroke, there has not yet been direct confirmation of this.

The demonstration of accelerated neurogenesis after cerebral ischemia has led to the proposal that newly formed neurons may contribute to functional recovery after stroke (Liu et al., 1998; Jiang et al., 2001; Jin et al., 2001). Factors regulating neurogenesis remain poorly understood, but the essential role of astrocytes in neuronal differentiation during development suggests that astrocytes may similarly be important in regulating neurogenesis. In support of this idea, it has recently been shown that astrocytes induce neurogenesis from adult neural stem cells in culture (Song et al., 2002). Astrocytes are also likely to play a central role in angiogenesis after stroke. Astrocytes promote angiogenesis through the release of VEGF, angiopoietin-1, and epoxyeicosatrienoic acids (Salhia et al., 2000; Acker et al., 2001; Chow et al., 2001; Zhang and Harder, 2002). Studies of brain development show a close relationship between astrocyte expression of angiogenic factors and microvessel development (Acker et al., 2001), and studies of brain injury confirm a temporal and spatial correlation between reactive astrocytosis, VEGF immunoreactivity, and microvascular density.

7 Microglia Effects on Ischemic Injury

Microglia are the resident macrophages of the CNS. They are derived from bone marrow precursors and have a slow interchange with the circulating macrophage pool (Perry and Gordon, 1988; Kaur et al., 2001). Activated microglia express surface markers similar to macrophages, but microglia have faster reaction time, better mobility, and greater proliferation capacity than macrophages (Giulian et al., 1995; Lyons et al., 2000). Microglia in the normal brain have a highly branched "ramified" morphology that can rapidly transform into activated, amoeboid morphology in response to stressors such as ischemia (Morioka et al., 1991; Kreutzberg, 1996; Raivich et al., 1998). Microglia undergo massive activation after stroke that remains detectable for several weeks (Jorgensen et al., 1993). Ischemia-induced changes in extracellular ion composition are thought to be a trigger of microglial activation (Boucsein et al., 2000; Kato and Walz, 2000; Schipke et al., 2002). Microglial activation is also induced and modulated by glutamate binding to glutamate receptors (Gottlieb and Matute, 1997; Biber et al., 1999; Noda et al., 2000; Tikka and Koistinaho, 2001; Tikka et al., 2001; Taylor et al., 2003).

7.1 Neurotoxic Effects of Activated Microglia

In vivo and in vitro studies show that microglial activation precedes neuronal death (Morioka et al., 1991; Gehrmann et al., 1992; Tikka and Koistinaho, 2001), and the timing and location of microglial activation correlates with neuronal death (Morioka et al., 1991, 1992; Jorgensen et al., 1993; Zhang and Fedoroff, 1996). Inhibition of microglial activation improves neuronal survival after excitotoxic and ischemic insults (Rogove and Tsirka, 1998; Yrjanheikki et al., 1999). Activated microglia can promote neuronal death by releasing glutamate (Piani et al., 1991) and the NMDA receptor modulator D-serine (Wu et al., 2004). Microglia also release cytokines, free radicals, and proteases that can affect neuronal viability directly or by modulating function of other brain cells (*Table 9-1*). For example, microglia show early expression of IL-1β after excitotoxic or ischemic insults (Davies et al., 1999; Pearson et al., 1999). Inhibition of IL-1β reduces and the administration of IL-1β increases neuronal death in these settings (Loddick et al., 1996; Lawrence et al., 1998; Davies et al., 1999; Pearson et al., 1999). Activated microglia release nitric oxide through expression of iNOS and may also promote neuron death by release of MMP-9 (Kauppinen and

Table 9-1
Molecules expressed and produced by microglia

Class	Name	References
Surface antigens	MHC I and II, CD4, CD8, CD11b, CD68, ICAM-1	Perry and Gordon (1987), Morioka et al. (1992), Schroeter et al. (2001)
Cytokines and chemokines	ILs (IL-1α and β, -3, -5, -6, -8, -10, -12, -16), TNF-α, M-CSF, GM-CSF, MIP-1, PGs (D_2, E_2, F_2), RANTES	Giulian and Ingeman (1988), Gebicke-Haerter et al. (1989), Thery et al. (1990), Hetier et al. (1991), Sawada et al. (1993), Chao et al. (1995b), Gottschall et al. (1995), Murphy et al. (1995), Sheng et al. (1995), Walker et al. (1995), Aloisi et al. (1997), Hu et al. (1999), Janabi et al. (1999), Schwab et al. (2001)
Enzymes	COX-2, cPLA2, iNOS, ICE, MAPKs (p38, p44/42, JNK), PYK2, PKCs (βI, βII, θ, δ, η, ζ, ι), t-PA, u-PA, PA, MMPs (MMP-1, -2, -3, -9)	Nakajima et al. (1992a), Nakajima et al. (1992b), Gottschall et al. (1995), Paakkari and Lindsberg (1995), Bhat et al. (1996), Clemens et al. (1996), Fiebich et al. (1996), Maeda and Sobel (1996), Tsirka et al. (1997), Bhat et al. (1998), Zhang et al. (1998), Koponen et al. (2000), Tian et al. (2000)
Receptors and transporters	Glutamate receptors (GluR4, NR1, mGluR3, mGluT5), glutamate transporter (EAAT2/GLT-1), Immunoglobulin G receptor, thrombin receptors, purinergic receptors	Kondo et al. (1995), Ferrari et al. (1997), Gottlieb and Matute (1997), Biber et al. (1999), Moller et al. (2000), Noda et al. (2000)
Free radicals	Nitric oxide, peroxynitrite, superoxide anions	Chao et al. (1992), Chao et al. (1995a), Colton et al. (1996)
Neurotoxins	AA, APP, glutamate, quinolinic acid, L- and D-serine, unidentified excitotoxin	Piani et al. (1991), Banati et al. (1993), Giulian et al. (1993), Minghetti and Levi (1995), Heyes et al. (1996), Wu et al. (2004)
Growth and trophic factors	NGF, BFGF, TGF-α and -β, GDNF, NT-3, IGF-I	Mallat et al. (1989), Shimojo et al. (1991), Walker et al. (1995), Elkabes et al. (1996), Honda et al. (1999), O'Donnell et al. (2002)
Ion channels	Inward and outward rectifying K-channels	Lyons et al. (2000)

AA, arachidonic acid; APP, amyloid precursor protein; BFGF, basic fibroblast growth factor; COX-2, cyclooxygenase; cPLA2, cytoplasmic phospholipase A; iNOS, inducible nitric oxide synthase; GDNF, glial cell-derived neurotrophic factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; ICAM-1, intracellular adhesion molecule-1; ICE, interleukin-1 converting enzyme; IGF-I, insulin growth factor I; IL, interleukin; MAPK, mitogen-activated protein kinase; M-CSF, macrophage colony-stimulating factor; MHC, major histocompatibility complex; MIP-1, macrophage inflammatory protein-1; MMP, matrix metalloproteases; NGF, nerve growth factor; NT-3, neurotrophin-3; PA, plasminogen activator; PG, prostaglandin; PKC, protein kinase C; PYK2, proline rich protein tyrosine kinase 2; RANTES, regulation on activation normal T-cell expressed and secreted; TGF, transforming growth factor; TNF, tumor necrosis factor; t-PA, tissue-specific plasminogen activator; u-PA, urokinase-plasminogen activator

Swanson, 2004). Phagocytosis of neurons by activated microglia is commonly observed after brain ischemia, but this is likely a result rather than a cause of neuron death because microglia have not been observed to engulf healthy neurons (Kreutzberg, 1996; Streit, 2002). For example, spreading depression in the absence of ischemia does not lead into neuronal damage even though it does induce microglial activation (Gehrmann et al., 1993). Recent studies suggest that neurogenesis after ischemia is also impaired by activated microglia. Inhibition of microglial activation improves neurogenesis and facilitates survival of engrafted progenitor cells in rodent models (Ekdahl et al., 2003; Lacza et al., 2003; Sasaki et al., 2003).

7.2 Trophic and Neuroprotective Functions of Microglia

In addition to their cytotoxic actions, microglia can release a number of trophic factors that promote tissue repair and regeneration (Kreutzberg, 1996) (*Table 9-1*). These factors may be important determinants of outcome after brain ischemia. In cell culture studies, the presence of resting microglia enhances neuron survival and neurite outgrowth (Zhang and Fedoroff, 1996). Resting microglia also improve astrocyte survival in a cell culture model of ischemia through release of GDNF (Lee et al., 2004). In addition, microglia are capable of high-affinity, high-velocity glutamate uptake via their glutamate transporters (Kondo et al., 1995; Lopez-Redondo et al., 2000; Nakajima et al., 2001; van Landeghem et al., 2001), but the biological significance of this during ischemic conditions remains to be established.

In vivo, secretion of the neurotrophic factors GDNF and BDNF by activated microglia promotes dopaminergic sprouting in a model of striatal injury (Batchelor et al., 1999). Similarly, transplantation studies show that engrafted microglial cells in injured spinal cord promote neurite growth, although the mechanism remains to be established (Rabchevsky and Streit, 1997). Microglia increase expression of neurotrophic factors such as BDNF, IL-6, and TGF-β after ischemia (Lehrmann et al., 1998; Suzuki et al., 1999; Lee et al., 2002); however, there is as yet no direct evidence that microglia promote neuronal survival in this setting.

7.3 Interactions Between Microglia and Astrocytes

Astrocytes and microglia are both capable of influencing one another (Kim, 1996). Microglia in the vicinity of an ischemic infarct secrete cytokines and chemokines that recruit additional microglia to the infarct site (Kreutzberg, 1996). Microglial activation also induces astrocytic proinflammatory response (Giulian and Baker, 1985; Kreutzberg, 1996; Tanaka et al., 1999a; Hailer et al., 2001), and astrocytic glutamate transport is affected by nitric oxide and TNF-α released by activated microglia (Trotti et al., 1996; Gegelashvili and Schousboe, 1997; Bezzi et al., 2001). Conversely, astrocytes influence microglial activation and migration. Microglia in coculture with astrocytes or astrocyte-conditioned medium maintain the resting, ramified morphology, but assume the activated, amoeboid morphology in the absence of astrocytes (Giulian et al., 1995; Kloss et al., 1997; Tanaka et al., 1999a). This affect is mediated by TGF-β, M-CSF, and GM-CSF, as evidenced by the effects of neutralizing antibodies (Schilling et al., 2001). Astrocytes also stimulate microglial proliferation and migration after injury (O'Donnell et al., 2002; Zhang et al., 2003).

8 Summary

Astrocytes and microglia have dynamic interactions with neurons and with each other that influence outcome from stroke and other insults. These interactions involve energy metabolism, redox metabolism, neurotransmitter uptake and release, trophic factor support, and formation of toxic intermediaries. The complexity of these interactions precludes a simple dichotomous classification of glia as positive or negative modulators of brain injury. By the same token, however, these complexities suggest that specific interactions between these cell types may be targeted for therapeutic intervention.

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10 Cerebral Endothelial Cell Reaction to Ischemic Insults

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Abstract: Clinical as well as basic research in the past decades has revealed the crucial importance of cerebral endothelial cell (CEC) dysfunction contributing to secondary injury following primary brain ischemic insults. Multiple postischemic events including inflammation, blood–brain barrier (BBB) breakdown, vasogenic brain edema, and hemorrhagic transformation are known to worsen the clinical outcomes of stroke patients, all of which are closely associated with CEC dysfunction. Oxidative stress with excessive production of reactive oxygen species (ROS) or free radicals has been considered to be one of the major factors in causing ischemic brain damages. In this chapter, we first discuss the sources of ROS and its contribution to vascular injury as well as recent progresses using antioxidant enzymes, most notably SOD and catalase, to attenuate ischemic injury. Cerebral ischemia results in damages of the brain vasculature with breaching of the normally impermeable BBB, thereby leading to vasogenic brain edema after stroke. We then review the potential importance of several proteins known to be involved in BBB breakdown after stroke, including matrix metalloproteinases (MMPs), endothelin, vascular endothelial growth factor (VEGF), angiopoietin, and nitric oxide synthase (NOS). Postischemic inflammatory reaction is another detrimental event causing secondary vascular injury, and thus exacerbating the primary ischemic damage. CEC dysfunction may promote adherence and infiltration of inflammatory cells, including neutrophils, macrophages, lymphocytes, and platelets. Therefore, cell and protein mediators contributing to postischemic inflammation are discussed. We also discuss several redox-sensitive transcription factors including hypoxia-inducible factor-1 (HIF-1), nuclear factor kappa-B (NF-κB), and activator protein-1 (AP-1) that are activated after cerebral ischemia to alter CEC viability and postischemic angiogenesis. Finally, potential therapeutic strategies such as statins, ischemic preconditioning (IPC) or LPS preconditioning, and prostaglandins that have been shown to exert protective effects in the settings of cerebral ischemia via mechanisms involving endothelial functions are emphasized.

List of Abbreviations: AIF, apoptosis-inducing factor; AP-1, activator protein-1; Ang-1, angiopoietin-1; ATM, ataxia telangectasia; BBB, blood-brain barrier; BDNF, brain-derived neutrophic factor; CEC, cerebral endothelial cells; CNS, central nervous system; COX-1, cycloxygenase-1; CRP, C-reactive protein; Cu/Zn-SOD, copper/zinc superoxide dismutase; eNOS, endothelial nitric oxide synthase; EPO, erythropoietin; ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; FRA, Fos-related antigen; GC, guanylate cyclase; GR, glucocorticoid receptor; GSNO, S-nitrosoglutathione; HIF, hypoxia-inducible factor; HMG, 3-hydroxy-3-methylglutaryl; HO-1, heme oxygenase-1; HRE, hypoxia-response element; ICAM-1, intercellular adhesion molecule-1; iNOS, inducible nitric oxide synthase; IPC, ischemic preconditioning; JNK, c-Jun N-terminal kinase; L-NA, N(omega)-nitro-L-arginine; L-NAME, N(G)-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MCAO, middle cerebral artery occlusion; MMP, matrix metalloproteinase; Mn-SOD, manganese superoxide dismutase; NAC, N-acetylcysteine; NAD, nicotinamide adenine dinucleotide; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor kappa-B; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; OGD, oxygen-glucose deprivation; PARP-1, poly(ADP-ribose) polymerase-1; PGI_2, prostacyclin; PGIS, prostacyclin synthase; PI3K, phosphatidylinositol 3-kinase; PPAR-γ, peroxisome proliferators-activated receptor-gamma; rhVEGF, recombinant human vascular endothelial growth factor; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; Stat, signal transducer and activator of transcription; tPA, tissue plasminogen activator; TSP-1, thrombospondin-1; TWEAK, tumor necrosis factor-like weak inducer of apoptosis; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor

1 Introduction

Cerebral ischemia causes vascular damages that may trigger multiple secondary brain injury processes, including blood–brain barrier (BBB) breakdown, vasogenic brain edema, and hemorrhagic transformation, ultimately worsening clinical outcomes in stroke patients. Therapeutic interventions directed at vascular protection need to be developed in addition to neuroprotective regimens. In this chapter, we review the neurochemical mechanisms that underlie ischemia-induced damages to cerebral vasculature. We will

also address therapeutic strategies directed at preserving viability and function of cerebral endothelial cells (CECs).

2 Oxidative Injuries to Cerebral Vasculature Following Ischemia–Reperfusion

2.1 Sources of Reactive Oxygen Species

Brain ischemia results in insufficient blood supply of oxygen, glucose, and nutrients, leading ultimately to tissue injury. Oxidative stress with excessive production of reactive oxygen species (ROS) or free radicals has been considered to be one of the major factors in causing ischemic brain injury. ROS generated by cerebral ischemia directly attack several subcellular elements, including membranes, proteins, and nucleic acids. The endothelial cell is a primary target of oxidative damage following ischemic insults. ROS-mediated changes may be particularly significant because of the multitude of changes closely associated with endothelial dysfunction or death. Postischemic generation of ROS in CECs may include superoxide anion, hydroxyl radicals, nitric oxide (NO), and peroxynitrite (Chan, 1996). Superoxide anion generation has been detected in the brain during focal ischemia and reperfusion in rats (Fabian et al., 1995) as well as in gerbils (Yamaguchi et al., 1998). Several mechanisms have been proposed to be responsible for the generation of superoxide anion. Cerebral ischemia induced the conversion of xanthine oxidase from a nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenase to an oxygen-dependent superoxide-producing oxidase in a four-vessel occlusion method of global brain ischemia in rats (Kinuta et al., 1989). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is another superoxide-generating enzyme. The extent of ischemic brain injury after transient middle cerebral artery occlusion (MCAO) was reduced in mice lacking a functional phagocytic NADPH oxidase (Walder et al., 1997). Superoxide anion produced by activated macrophages also leads to mitochondrial dysfunction and apoptosis in endothelial cells (Madesh et al., 2005). Fatty acids may also be a major source of superoxide anion. Superoxide anion formation in newborn pig brains during reperfusion after global ischemia was attributed to activation of cyclooxygenase metabolism of arachidonic acid (Armstead et al., 1988). Together these findings suggest that superoxide anion may be generated via multiple pathways.

2.2 ROS-Mediated Vascular Injury

ROS contribute to BBB breakdown and vasogenic brain edema after cerebral ischemia–reperfusion. Superoxide dismutase (SOD) is a superoxide anion scavenger enzyme that converts superoxide anion to hydrogen peroxide. Topical application of SOD to the ischemic cortex in a permanent MCAO model substantially reduced the formation of superoxide anion. Liposome-entrapped SOD also significantly attenuated the extent of brain edema (Chan et al., 1990). Superoxide anion may also affect BBB integrity. After ischemia, an increase in BBB permeability to macromolecules was reduced by pretreatment with SOD and catalase (Nelson et al., 1992). Conceivably, ROS may damage CECs, leading to their enhanced interaction with inflammatory cells including phagocytes and neutrophils to cause secondary vascular injury and further increase BBB permeability. Interestingly, these inflammatory cells also contribute to postischemic production of superoxide anion. As referred to earlier, NADPH oxidase in phagocytes is a major enzyme in the generation of superoxide anion. Mutant mice with a dysfunctional phagocytic NADPH oxidase had smaller infarct volumes in a transient MCAO model (Walder et al., 1997). Neutrophil is another important source of superoxide anion especially during reperfusion. In a transient MCAO model in rats, treatment with an anti-CD18 antibody that reduced the extent of neutrophil accumulation in the ischemic region significantly attenuated superoxide anion production during reperfusion (Fabian and Kent, 1999). These results indicate that oxidative stress following ischemia–reperfusion and the subsequent infiltration of inflammatory cells trigger progressive endothelial events, perpetuating BBB breakdown and vasogenic brain edema in a vicious circle.

Despite being a primary target of oxidative attack, endothelial cells themselves have been proposed to be a significant source of ROS. After ischemia–reperfusion, superoxide anion was identified in the meninges in association with blood vessels, located primarily in the extracellular space and in endothelial as well as vascular smooth muscle cells. This oxidative process could be attenuated by SOD (Kontos et al., 1992). Production of superoxide anion was also demonstrated in the vascular lumen following transient MCAO and reperfusion. Superoxide anion was detected within arterial, capillary, and venular lumen, suggesting a progressive increase in intraluminal generation of this oxygen radical following ischemia–reperfusion (Mori et al., 1999). ROS including superoxide anion were produced in excessive amounts in CECs in primary cultures derived from piglet brains following anoxia/reoxygenation with the xanthine oxidase pathway being the primary source. ROS, generated under this condition, exerted their cytotoxic action on the very cells that produced them (Beetsch et al., 1998).

2.3 Superoxide Dismutase

Recent technical improvements in generating transgenic animals provide direct evidence confirming the involvement of superoxide anion in ischemic brain injury. Transgenic mice overexpressing human copper/zinc superoxide dismutase (Cu/Zn-SOD) were highly resistant to reperfusion injury after focal cerebral ischemia (Yang et al., 1994). In contrast, reduction in Cu/Zn-SOD activity aggravated neuronal cell injury and edema formation after transient focal cerebral ischemia (Kondo et al., 1997). Cu/Zn-SOD overexpression in transgenic mice (Murakami et al., 1997) or rats (Chan et al., 1998) had reduced hippocampal injury after global ischemia. In contrast, mice with homozygous Cu/Zn-SOD knockout exhibited significant exacerbation of delayed injury in the vulnerable hippocampal CA1 region with increased superoxide anion production after transient global ischemia (Kawase et al., 1999). Similar to Cu/Zn-SOD knockout mice, mutant mice deficient in manganese superoxide dismutase (Mn-SOD), which is localized in mitochondria, were also more susceptible to ischemia-induced oxidative stress. In the heterozygous Mn-SOD knockout mice, superoxide anion production was accentuated after focal cerebral ischemia, leading to larger infarct volumes and more severe neurological deficit (Murakami et al., 1998). Apart from heightened oxidative stress, mice deficient in Mn-SOD showed accelerated release of mitochondrial cytochrome *c* and DNA fragmentation after permanent MCAO, indicating Mn-SOD may exert a protective action against ischemia-induced, mitochondria-dependent apoptosis (Fujimura et al., 1999).

2.4 Nitric Oxide

Another reactive species that may be involved in ischemic brain damage is NO. In cultured bovine CECs, oxygen–glucose deprivation (OGD) that mimics ischemic injury in vitro induced apoptosis with formation of nitrotyrosine suggesting NO and its derivatives, particularly peroxynitrite, a reactive nitrogen species (RNS), may play an important role in ischemia-induced CEC death (Xu et al., 2000). NO may be synthesized from L-arginine and molecular oxygen catalyzed by nitric oxide synthase (NOS). Three isoforms of NOS have been identified, the neuronal NOS (nNOS or NOSI), the inducible NOS (iNOS or NOSII), and the endothelial NOS (eNOS or NOSIII). Surges of NO and superoxide anion generation upon reperfusion have been demonstrated with concomitant generation of peroxynitrite, an RNS, which interacts with tyrosine residues on proteins to form nitrotyrosine, resulting in protein dysfunction (Beckman et al., 1990). During reperfusion, intense superoxide, NO, and peroxynitrite formation on microvessels and surrounding astrocytic endfeet led to cerebral hemorrhage by disrupting microvascular integrity (Gursoy-Ozdemir et al., 2004). In a mouse model of MCAO with reperfusion, infarct volumes were significantly smaller in animals treated with a nonselective NOS inhibitor, *N*-ω-nitro-L-arginine (L-NA), upon reperfusion. NOS inhibition also significantly reduced vascular damage as indicated by a reduction in BBB permeability (Gursoy-Ozdemir et al., 2000). Another mode of NO-dependent endothelial injury may be derived from induction of iNOS during the postischemic inflammatory response in which CECs are one of the primary targets. After OGD in vitro, bovine CEC death was accompanied by upregulation of iNOS

mRNA and protein expression and an increase in cellular nitrotyrosine content. Application of NOS inhibitors and an NO scavenger rescued CECs from OGD-induced cell death (Xu et al., 2000). Suppression of iNOS by stable overexpression of its antisense RNA also prevented CECs from inflammatory cytokine-induced apoptosis (Yang et al., 2005a). Despite potential toxicity of massive NO derived from nNOS or iNOS after ischemia–reperfusion, NO production from eNOS, in contrast, appears to decrease ischemic injury via induction of vasodilatation and modulation of cerebral blood flow. eNOS upregulation by chronic simvastatin treatment or infusion of the NOS substrate L-arginine significantly increased the absolute brain blood flow. Furthermore, pretreatment with simvastatin enhanced blood flow within the ischemic brain region after MCAO, suggesting that eNOS activity is critical for compensatory blood flow augmentation in the ischemic brain (Yamada et al., 2000).

Although the development of NOS inhibitors aided in the identification of selective NO actions during ischemia–reperfusion, specific inhibition of each of the three NOS isoforms has not been achieved to clearly define their respective roles in ischemic vascular injury. Applications of knockout mice deficient in each NOS isoform provided unambiguous results regarding exact sources of NO produced by each NOS isoform after stroke. Following cerebral ischemia, a rapid increase in NO occurred in nNOS(–/–) mice peaking after only 10 min, whereas the production of NO in the eNOS(–/–) mice paralleled that in the wild type with a progressive increase over 20 min, suggesting progressive accumulation of NO from nNOS following cerebral ischemia (Wei et al., 1999). Binding of radiolabeled L-NG-nitroarginine, suggestive of NOS activation, was significantly increased in the MCA territory during ischemia and the first 3 h of reperfusion in both the wild-type and the eNOS-null mouse. In contrast, the binding was low and only a very small increase was observed after ischemia in the nNOS-deficient mouse, indicating that cerebral ischemia increased nNOS, but not eNOS, activity that may contribute to pathogenesis of postischemic BBB breakdown and vasogenic brain edema after cerebral ischemia–reperfusion (Hara et al., 1997). Using mice with selective NOS isoform knockout, detrimental or salutary roles of NO derived from various NOS isoform during ischemia–reperfusion have been explored. In nNOS(–/–) mice, infarct volumes were smaller 24 and 72 h after MCAO accompanied by less prominent neurological deficit. However, infarct volumes in the mutant became larger when nitro-L-arginine, a nonselective NOS inhibitor, was administered to inhibit NO synthesis. These findings suggest that neuronal NO production by nNOS is detrimental while vascular NO generation by eNOS is salutary after cerebral ischemia–reperfusion (Huang et al., 1994).

3 Protein Mediators of Ischemia-Induced BBB Breakdown

After cerebral ischemia–reperfusion, brain vasculature becomes leaky and unstable, and the normally impermeable BBB breaks down, contributing to the development of vasogenic brain edema after stroke. Several proteins are known to be involved in causing BBB breakdown after cerebral ischemia–reperfusion. These include matrix metalloproteinases (MMPs), endothelin, vascular endothelial growth factor (VEGF), angiopoietin, and NOS. The varied roles of NOS isoforms in altering BBB function have been reviewed above. MMPs are described in *Sect. 4* (Postischemic Inflammatory Reaction).

3.1 Endothelin

Endothelin is a strong vasoconstrictor that may mediate vasoconstriction. Vasoconstriction under ischemic conditions could theoretically aggravate ischemic neuronal damage. Stroke patients have elevated plasma and cerebrospinal fluid levels of endothelin-1 (ET-1). Endothelin immunoreactivity was found in endothelial cells of hippocampal microvessels and in astrocytes, microglia, and in some axonal endings after transient global ischemia in rats (Gajkowska and Mossakowski, 1995). In a rabbit model of transient MCAO, concentrations of ET-1 in the ischemic brain and plasma were both significantly increased (Bian et al., 1994). Despite the heightened expression of endothelin after cerebral ischemia, its pathological role in ischemic vascular injury remains to be fully defined. Transgenic mice overexpressing ET-1 in astrocytes showed more severe neurological deficit, larger infarct volumes, increased BBB breakdown, and brain water

content after transient MCAO, suggesting that astrocytic ET-1 has deleterious effects on water homeostasis and BBB integrity, contributing to ischemic vascular injury (Lo et al., 2005).

3.2 Vascular Endothelial Growth Factor

VEGF is a secreted mitogen angiogenic factor with potent action in enhancing vascular permeability. The role of VEGF in causing BBB breakdown following cerebral ischemia remains controversial. In a rat model of focal cerebral embolic ischemia, late administration of recombinant human VEGF (rhVEGF) to ischemic rats enhanced angiogenesis in the ischemic penumbra and markedly improved neurological recovery. However, early administration of rhVEGF significantly increased BBB leakage and hemorrhagic transformation with enlarged ischemic lesions (Zhang et al., 2000). Single intracerebroventricular injection of VEGF was effective in reducing BBB breakdown in a rat MCAO model. This protective effect of VEGF appeared to involve activation of phosphatidylinositol-3-kinase (PI3K)/Akt-signaling cascades (Kaya et al., 2005). In contrast to the intracerebroventricular route, intravenous VEGF increased the infarct volumes (Kaya et al., 2005).

3.3 Angiopoietin-1

Angiopoietin-1 (Ang-1) is a ligand for the endothelial specific receptor tyrosine kinase, Tie2, which preserves the integrity of the adult peripheral vasculature. In a mice model of embolic MCAO, recombinant adenoviruses expressing Ang-1 or the recombinant Ang-1 protein reduced BBB breakdown and infarct volumes. Ang-1 administration also reduced BBB breakdown caused by rhVEGF infusion into ischemic mice brains (Zhang et al., 2002a). These findings, together with the observations of temporal and spatial expression profiles of VEGF, Ang-1, and their cognate receptors after cerebral ischemia (Lin et al., 2000, 2001), suggest that VEGF and Ang-1 may alter BBB permeability in the ischemic core, whereas upregulation of VEGF/VEGF receptors and Ang-1/Tie2 in the penumbra may promote neovascularization in ischemic brains (Zhang et al., 2002b).

4 Postischemic Inflammatory Reaction

Postischemic inflammatory reaction may cause secondary vascular injury exacerbating the primary ischemic insult. CEC dysfunction and injury may promote adherence and infiltration of inflammatory cells, including neutrophils, macrophage, lymphocytes, and platelets, contributing to progressive vascular injury characterized by BBB breakdown and vasogenic brain edema.

4.1 Inflammatory Cells

In the mouse bilateral common carotid artery occlusion model with reperfusion, rolling and firm adhesion of platelets in cerebral venules were detected but preceded by a more intense recruitment of rolling and adherent leukocytes (Ishikawa et al., 2003). During cerebral ischemia, endothelial dysfunction may enhance leukocyte-endothelial interaction, facilitating BBB breakdown. As discussed in a previous section on oxidative vascular injury, inflammatory cells including neutrophils (Fabian and Kent, 1999) and phagocytes (Walder et al., 1997) are major sources of superoxide anion during ischemia and reperfusion.

4.2 Inflammatory Mediators

To a large extent, inflammatory mediators are derived from inflammatory cells. Inflammatory mediators including ROS, proteases, cytokines, and eicosanoids also play major roles in ischemic vascular injury.

An increase in the expression of MMP-9 was detected in CECs and infiltrating neutrophils in response to cerebral focal ischemia (Romanic et al., 1998). Reduced ischemic vascular injury in homozygous MMP-9 knockout mice and chimeric knockouts lacking MMP-9 either in leukocytes or in resident brain cells suggest that leukocytes, most likely neutrophils, are a key cellular source of MMP-9, which in turn promotes leukocyte recruitment and causes BBB breakdown secondary to microvascular basal lamina proteolysis, thus ultimately contributing to neuronal injury after transient focal ischemia (Gidday et al., 2005). MMP-9, however, may be important in other actions including remodeling of neural structure in regenerative processes to restore function after ischemic insult. Inhibition of MMP-9 expression in the subacute stage (7 days after ischemic insult) impaired functional recovery (Zhao et al., 2006). Ischemia-reperfusion may trigger an acute phase response resulting in a rise in plasma concentrations of C-reactive protein (CRP), contributing to postischemic inflammatory reaction. A significant increase in circulating levels of CRP was observed in an in vivo rat brain ischemia model of MCAO. In mouse CECs, CRP induced intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression, suggesting that CRP may be involved directly in the development of inflammation in response to cerebral ischemia (Zhang et al., 2006). NO derived from various isoforms of NOS may affect postischemic inflammatory response. Recent data indicate a regulatory role for NO in initial leukocyte-endothelial interactions in the cerebral microcirculation under ischemic conditions. Endogenous NO production during and after transient focal cerebral ischemia can limit the process of neutrophil infiltration and its deleterious consequences. Application of the nonselective NOS inhibitor L-NAME after the onset of ischemia increased the cortical infarct volume and myeloperoxidase activity, an index of neutrophil infiltration, indicating that after transient focal ischemia early NO production may exert a protective effect by modulating neutrophil infiltration in addition to its well-known vasodilatory action (Batteur-Parmentier et al., 2000). However, massive NO production derived from iNOS during later inflammatory phase likely contributes to further vascular damages. *Figure 10-1* summarizes ROS and protein mediators of ischemic vascular injury.

5 Transcription Factor Activation and Alteration of Gene Expression after Ischemia–Reperfusion

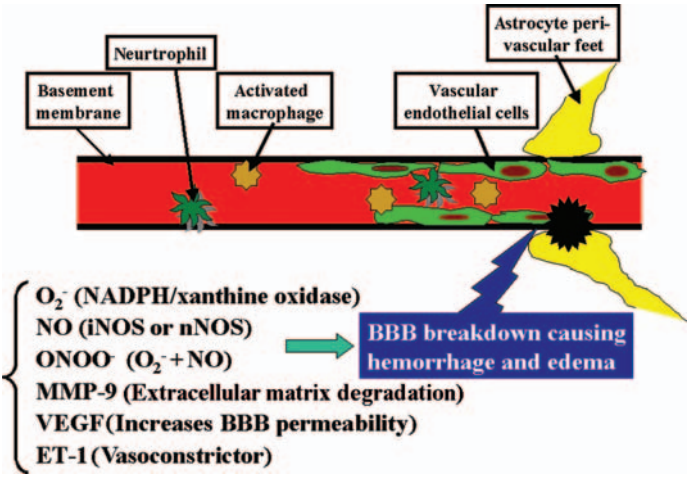
Several transcription factors and related signal transduction pathways may be activated after cerebral ischemia to alter CEC viability (*Figure 10-2*). These transcription factors include hypoxia-inducible factor-1 (HIF-1), nuclear factor kappa-B (NF-κB), and activator protein-1 (AP-1).

5.1 HIF

HIF-1 is a key regulator of mammalian oxygen homeostasis initially identified as a DNA-binding protein that recognizes an essential DNA sequence in the hypoxia-inducible enhancer of the erythropoietin (EPO) gene (Semenza and Wang, 1992). HIF-1 is a heterodimeric protein complex consisting of alpha (HIF-1α) and beta (HIF-1β) subunits. HIF-1α is inducible by a decrease in tissue or cellular oxygen tension. Binding of HIF-1α and HIF-1β forms a dimer to transcriptionally activate a large number of genes that contain the consensus sequence 5′-RCGTG-3′, the *cis* hypoxia-response element (HRE), in their promoter or enhancer regions (Semenza, 2001a, b). HIF-1α expression is tightly regulated by cellular oxygen tension (Wang et al., 1995), whereas the expression of HIF-1β subunit is oxygen independent. Hypoxic conditions prevent ubiquitination and subsequent proteasomal degradation of HIF-1α (Kallio et al., 1999), causing accumulation of this basic helix-loop-helix transcription factor in response to low oxygen tension (Semenza and Wang, 1992; Wang et al., 1995). The downstream genes that can be transactivated by HIF-1 include those involved in angiogenesis such as VEGF and EPO. Angiogenic factors stimulate the growth and differentiation of erythroid progenitor cells, as well as those involved in glucose metabolism like glucose transporters and glycolytic enzymes capable of increasing anaerobic ATP synthesis (Semenza, 1998). Following permanent MCAO in rats, mRNAs encoding HIF-1α as well as genes downstream of HIF-1, including the glucose

Figure 10-1

ROS and protein mediators contributing to postischemic endothelial injury. Endothelial cell dysfunction and death are caused by ROS and protein mediators following cerebral ischemia-reperfusion. The superoxide anion (O_2^-) may be derived from NADPH oxidase, xanthine oxidase, or cyclooxygenase metabolism of arachidonic acid in activated macrophages, neutrophils, or even CECs per se. Stroke-induced activation of nNOS in neurons and iNOS in macrophages and CECs with massive production of NO may also directly damage vasculature or, alternatively, cause indirect detrimental effects via interaction with superoxide anion to form peroxynitrite ($ONOO^-$). After primary insult by ROS, secretion of MMPs and production of VEGF further enhance breakdown of normally impermeable BBB, leading to vasogenic brain edema and hemorrhagic transformation. Inflammatory mediators may facilitate neutrophil, platelet, and macrophage adhesion to the endothelial surface, plugging capillaries, thereby causing secondary vascular injuries



transporter-1 gene and those of several glycolytic enzymes, were upregulated in the ischemic penumbra (Bergeron et al., 1999). VEGF and its cognate receptor (VEGF-R) were upregulated in the ischemic penumbra after MCAO (Marti et al., 2000). Similar to focal ischemia, transient global cerebral ischemia in the rat brain also resulted in activation of HIF-1 and VEGF upregulation in the vulnerable regions of cortex and hippocampus (Jin et al., 2000b). In a neonatal rat MCAO model, HIF-1α activation and VEGF upregulation were detected in neurons of the ischemic cortex (Mu et al., 2003). In the in vitro experimental paradigm, ROS production may also contribute to HIF-1 activation after prolonged hypoxia. Overexpression of cyclooxygenase-1 (COX-1) and prostacyclin synthase (PGIS) via adenovirus-mediated gene transfer augmented prostacyclin (PGI_2) production, resulting in the stabilization of HIF-1α in endothelial cells. PGI_2 reduced ROS production by attenuating NADPH oxidase activity under prolonged hypoxia (Chang et al., 2005).

Induction of HIF-1 conceivably may protect the brain from ischemic insults via multiple mechanisms. The first is cerebral angiogenesis. Postmortem evaluation of brain tissues from stroke patients has confirmed that cerebral ischemia causes active angiogenesis, which is more developed in the penumbra region. Higher blood vessel counts correlated with longer survival (Krupinski et al., 1994). Several factors may be responsible for postischemic angiogenesis. EPO and VEGF are pro-angiogenic factors downstream of HIF-1. VEGF expression after permanent MCAO was accompanied by an increase in the number of newly formed vessels at the infarct border (Marti et al., 2000). Exogenous application of HIF-1α-naked DNA was sufficient to induce the expression of VEGF with resultant increase in collateral circulation in a cerebral ischemic model (Matsuda et al., 2005). In addition to VEGF and EPO, endogenous NO derived from eNOS appears to enhance angiogenesis. A decrease in angiogenesis, as demonstrated by reduced CEC proliferation, vessel perimeter, and vascular density in the ischemic border, was evident in homozygous eNOS knockout mice compared with wild-type mice. The eNOS-deficient mice also exhibited a reduced response

Figure 10-2

Postischemic activation of transcription factors. Multiple transcription factors may be activated with subsequent induction of corresponding target genes after cerebral ischemia-reperfusion. Induction of HIF leads to expression of downstream genes, which is beneficial after stroke. HIF-dependent induction of VEGF and EPO that are antiapoptotic and facilitate postischemic neovascularization. Production of NO derived from eNOS, another HIF target gene, may also assist in preventing secondary vascular injury via induction of antiapoptotic and antioxidative genes. Heightened expression of glucose transporters (such as GLUT-1) and glycolytic enzymes, respectively, enhances glucose uptake and anaerobic glycolysis with resultant increase in ATP production. Activation of NF-κB may induce expression of iNOS with massive production of NO causing nitrosative stress to affect endothelial cell viability and function. NF-κB may transactivate MMPs' expression contributing to BBB breaching and ICAM-1 expression, facilitating neutrophil adhesion. Another redox-sensitive transcription factor is AP-1 that consists of various combinations of protein components, including c-Jun, c-Fos, Jun-B, Jun-D, Fos-B, and DeltaFosB. Despite early activation during cerebral ischemia, the pathophysiological functions of expression of these "immediate early genes" remain to be fully defined

Transcription factors	Target genes	Pathophysiological outcomes
HIF	VEGF/EPO	Angiogenesis and antiapoptosis
	eNOS	Vasodilatation and antiapoptosis
	GLUT-1	Glucose↑ and ATP↑
	Glycolytic enzymes	Lactate↑ and ATP↑
NF-κB	ICAM-1	Neutrophil adhesion and inflammation
	iNOS	Massive NO and nitrosative stress
	MMP	BBB breakdown
AP-1(DeltaFosB)	Galectin-1?	Neurogenesis
AP-1(c-Fos)	?	Hypothermic neuroprotection
AP-1(other components)	?	Exacerbation of neurological deficits ?

to VEGF-induced angiogenesis (Chen et al., 2005a). Human eNOS is a hypoxia-inducible gene, under transcriptional regulation of two contiguous HRE sequences located in the eNOS promoter (Coulet et al., 2003). Systemic administration of exogenous NO donors to rats 24 h after ischemic insult also substantially enlarged vascular perimeters and increased the number of proliferated CECs. These effects were completely abolished by a soluble guanylate cyclase (GC) inhibitor and a neutralizing antibody against VEGF-R2, suggesting that NO may enhance angiogenesis in the ischemic brain via the GC/cGMP pathway (Zhang et al., 2003). Overall, these results demonstrate that induction of HIF-1 may activate multiple factors including VEGF, EPO, and eNOS to enhance postischemic angiogenesis.

Several other factors are differentially expressed after cerebral ischemia that may also assist in neovascularization in the adult ischemic brain. These include neuropilin-1 (Zhang et al., 2001), brain-specific angiogenesis inhibitor-1 and -2 (Kee et al., 2002), Ang-1, Ang-2 (Zhang et al., 2002b), Tie-1, and Tie-2 (Lin et al., 2000, 2001). For example, Ang-2 mRNA was upregulated 6 h after MCAO and was mainly observed in CECs in the penumbral and infarcted areas in close association with EC proliferation (Beck et al., 2000). However, hypoxia-driven Ang-2 expression appears to be independent of the HIF pathway (Pichiule et al., 2004), suggesting additional mechanisms are likely involved in postischemic angiogenesis. Notably, sublethal OGD in murine CECs increased the extent of methylation of the promoter region of thrombospondin-1 (TSP-1), an endogenous angiostatic factor that inhibits VEGF action and hence angiogenesis. OGD-induced DNA methylation in the TSP-1 promoter was accompanied by a concurrent decrease in TSP-1 mRNA and protein expression, whereas TSP-1 promoter demethylation after reoxygenation led to the restoration of TSP-1 gene expression (Hu et al., 2006). As an increase in DNA methylation was noted in the

ischemic brain after mild ischemia (Endres et al., 2000), these results raise the possibility that epigenetic regulation of gene expression may represent an additional mechanism in the regulation of postischemic angiogenesis.

Tolerance to cerebral ischemia may be achieved by preconditioning with sublethal stresses, such as ischemia or hypoxia, in which a decrease in oxygen tension may constitute a sublethal stress to induce tolerance. This is known as ischemic preconditioning (IPC). In addition to postischemic angiogenesis, HIF activation may in part underlie the IPC-dependent neuroprotection. Thus, normobaric hypoxia (8% O_2) performed 24 h before focal permanent ischemia in adult mice reduced infarct volumes with an increase in the nuclear content of HIF-1α and heightened expression of its target genes, including EPO and VEGF, suggesting the possibility of HIF-1-mediated increase in ischemic tolerance (Bernaudin et al., 2002). In transient focal cerebral ischemia in rats, IPC increased HIF-1 DNA-binding activity and EPO expression. Infusion of soluble EPO receptor significantly reduced IPC-dependent protection, suggesting EPO is involved in the development of ischemic tolerance (Prass et al., 2003). Preconditioning in glial cells with chemical reagents known to activate HIF-1 also attenuated metabolic insults induced by mitochondrial inhibition. Under this experimental paradigm, inhibition of HIF-1 completely abolished this preconditioning effect (Yang et al., 2005b). Vitamin E has been shown to confer protective effects against cerebral ischemia, possibly due to its antioxidant effects. Interestingly, vitamin E also induced the expression of HIF-1α and its target genes, including VEGF and hemeoxygenase-1 (Ho-1). The hypoxia response element on the VEGF promoter was responsible for the vitamin E-induced transcriptional activation of the VEGF gene (Zhang et al., 2004). Thus, increased vitamin E contents in brain tissue may elicit neuroprotective effects not only through scavenging oxidants, as previously reported, but also by transactivating HIF-1-dependent genes to protect brains from ischemic insults. In addition to its pro-angiogenic action, VEGF has also been shown to exert direct neuroprotective effects via the PI3K/Akt signal transduction system in an in vitro model of cerebral ischemia (Jin et al., 2000a). Overall, hypoxia-induced activation of HIF-1 and its downstream genes implicated in angiogenesis and neuroprotection may provide salutary effects in the setting of cerebral ischemia–reperfusion.

5.2 NF-κB

Reperfusion injury is mediated in part by upregulation of genes in CECs that encode inflammatory cytokines and adhesion molecules. NF-κB is a redox-sensitive transcription factor that may play a major role in the transactivation of these genes. NF-κB activation has been noted following focal cerebral ischemia–reperfusion in a redox-dependent manner (Salminen et al., 1995). NF-κB, consisting of p65 and p50 Rel proteins, was significantly increased 15 min after reperfusion in a rat model of transient MCAO; administration of *N*-acetylcysteine (NAC), the precursor of glutathione and an antioxidant, inhibited NF-κB activation and reduced the extent of cerebral infarction, thereby attenuating reperfusion injury (Carroll et al., 1998). Similar induction of NF-κB by hypoxia-reperfusion was also observed in CECs in culture. When CECs were subjected to hypoxia followed by reoxygenation, but not hypoxia alone, an NF-κB complex composed of p65 and p50 Rel proteins was rapidly activated within 15–30 min followed by heightened expression of the ICAM-1 gene 4 h later; both were blocked by the antioxidant pyrrolidine dithiocarbamate (Howard et al., 1998). The OGD-induced NF-κB activation may be causally related to the expression of the ataxia telangectasia (ATM) gene. OGD stimulated ATM gene expression in CECs as early as 1 h after OGD initiation. Antisense knockdown of ATM attenuated OGD-induced NF-κB activation and the subsequent expression of downstream genes, including the antiapoptotic gene c-IAP2, leading to enhanced CEC death (Yin et al., 2002). In addition, NF-κB is involved in cytokine-induced expression of iNOS (Xu et al., 1997) that may have contributed to OGD-induced protein nitration with accumulation of 3-nitrotyrosine in CECs, resulting in endothelial death (Xu et al., 2000). In rat forebrain slices, inhibition of NF-κB by pyrrolidine dithiocarbamate also suppressed glutamate-mediated, calcium-dependent iNOS induction after OGD (Cardenas et al., 2000). Hypoxia-reperfusion-induced NF-κB activation may therefore induce iNOS expression that leads to massive production of NO causing oxidative stress with resultant detrimental effects in CNS cells, including CECs in the brain.

MMPs are another group of NF-κB target genes. Lipopolysaccharide (LPS) activates MMP-2 in CECs through an NF-κB-dependent pathway (Kim and Koh, 2000). Intracerebral injection of tumor necrosis factor-like weak inducer of apoptosis (TWEAK) in wild-type mice activated the NF-κB pathway and induced MMP-9, causing BBB breakdown. However, TWEAK failed to increase MMP-9, activity or BBB permeability when injected into mice genetically deficient in the NF-κB family member p50 (Polavarapu et al., 2005). Hypoxia-reoxygenation-induced MMPs may contribute to the pathophysiology of cerebral ischemia by degrading matrix components in the neurovascular unit with increased BBB permeability. It has been demonstrated that MMPs may trigger caspase-mediated cytotoxicity in CECs after hypoxia–reoxygenation by disrupting cell–matrix interactions and homeostatic integrin signaling (Lee and Lo, 2004).

5.3 AP-1

AP-1 is the third major transcriptional factor that may be induced after ischemia–reperfusion. An increased expression of immediate-early genes including c-Fos and Jun-B with enhanced AP-1-binding activity was detected in the ischemic cortex in a rat MCAO model (An et al., 1993). Ischemia-induced Fos expression and AP-1 activity can be suppressed by an antisense oligodeoxynucleotide to c-Fos mRNA (Liu et al., 1994). Following ischemic injury induced by MCAO in rats, induction of both c-Fos and Fos-related antigen (FRA-2) in penumbral regions was observed that preceded upregulation of the classic injury-associated proteins expressed by astrocytes and microglia and in CECs of the core ischemic region (Butler and Pennypacker, 2004). A delayed induction of Jun-B was also observed that preceded CA1 neuronal death after global ischemia in the gerbil (Whitfield et al., 1999). Despite extensive studies, whether AP-1 activation is detrimental or salutary after cerebral ischemia remains to be reconciled (Akins et al., 1996). Fasting, shown to reduce ischemic brain injury (Yip et al., 1991) in a rat MCAO model, prolonged c-Fos expression in the same model (Lin et al., 1997), suggesting c-Fos expression may be salutary. Early c-Fos expression in vulnerable hippocampal CA1 neurons may account for neuroprotection by upregulating late expression of survival genes in a global ischemia model (Cho et al., 2001). In a transient focal ischemia model, a decrease in infarct volumes was accompanied by an increase in c-Fos immunostaining and AP-1-binding activity in the cortex after reperfusion in the hypothermic, but not the normothermic group, suggesting a probable neuroprotective effect of c-Fos under hypothermic conditions (Akaji et al., 2003). In a different study, induction of DeltaFosB, another AP-1 subunit, appears to promote the proliferation of quiescent neuronal precursor cells, thus enhancing neurogenesis after transient forebrain ischemia (Kurushima et al., 2005).

In conclusion, postischemic activation of the redox-sensitive transcription factors including HIF, NF-κB, and AP-1 with subsequent induction of respective downstream target genes may contribute to both beneficial as well as detrimental effects after cerebral ischemia–reperfusion. Manipulations of the upstream transcription factors may represent an effective measure to reduce ischemic brain damage and to enhance neurological recovery after cerebral ischemia in the future.

6 Mitogen-Activated Protein Kinase Signaling in the Regulation of Hypoxic Cell Death in CECs

The mitogen-activated protein kinase (MAPK) pathway is widely involved in hypoxia-induced cell death. In an in vitro model system, CEC death after hypoxia-reoxygenation is mediated by the p38 MAPK–caspase pathway, but not the extracellular signal-regulated kinase (ERK) or c-Jun N-terminal kinase (JNK) cascades. Interrelationship between p38 MAPK and caspase-3 has been established in a CEC death paradigm following hypoxia–reoxygenation (Lee and Lo, 2003). In human CECs in cultures, nuclear translocation of apoptosis-inducing factor (AIF) and cleavage of poly(ADP-ribose) polymerase-1 (PARP-1) were observed after intermittent hypoxia–reoxygenation to cause cell death. The caspase inhibitor blocked PARP-1 cleavage but did not affect AIF translocation and was only modestly cytoprotective. These findings together indicate that PARP-1 activation and PARP-1-dependent, caspase-independent nuclear translocation of AIF may contribute to apoptotic CEC death after ischemia–reperfusion (Zhang et al., 2005). In an MCAO

model in rats, phosphorylation of signal transducer and activator of transcription-3 (stat-3) has been demonstrated. Double immunostaining with antibodies against marker proteins specific for each cell type in CNS revealed phosphorylated stat-3 to be present exclusively in neurons and CECs only during postischemic reperfusion, but neither in astrocytes nor in microglia/macrophages (Suzuki et al., 2001). However, the effects of stat-3 activation on cell viability of neurons or CECs remain unclear.

7 Potential Therapeutic Strategies Directed at Ischemic Vascular Injury

Several therapeutic strategies have been shown to exert protective effects in the settings of cerebral ischemia via mechanisms involving endothelial functions. These include statins, IPC or LPS preconditioning, prostaglandins, and others.

7.1 Statins

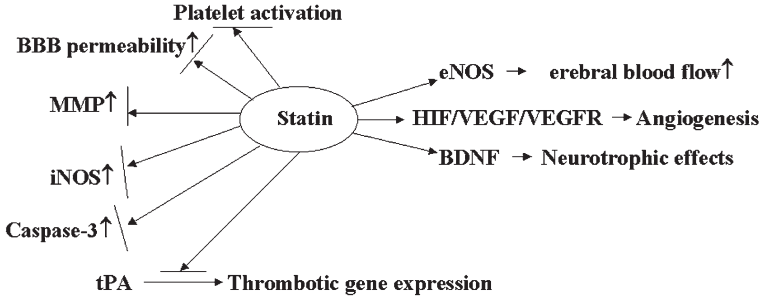
Statins are 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors, traditionally known as cholesterol-lowering agents. However, recent findings suggest that statins offer protection in cerebral ischemia via mechanisms independent of their effects on lowering cholesterol levels. Prophylactic treatment with statins augmented cerebral blood flow, reduced cerebral infarct volumes, and improved functional recovery in a rodent MCAO model. These beneficial effects were accompanied by eNOS upregulation but not associated with changes in serum cholesterol levels (Endres et al., 1998). Atorvastatin upregulated eNOS in thrombocytes, decreased platelet activation in vivo, and protected the brain from cerebral ischemia in normocholesterolemic mice (Laufs et al., 2000). Treatment with simvastatin after MCAO reduced ischemic brain injury, similar to that observed when simvastatin was administered before focal ischemia (Sironi et al., 2003). Other statins shown to have similar protective effects in various ischemic models include rosuvastatin (Laufs et al., 2002) and mevastatin (Amin-Hanjani et al., 2001). In addition to upregulating eNOS, atorvastatin may also increase VEGF, VEGFR2, and BDNF expression in the ischemic penumbra; numbers of migrating neurons, developmental neurons, and synaptophysin-positive cells as well as indices of angiogenesis were significantly increased in the atorvastatin treatment group, demonstrating that atorvastatin promoted angiogenesis, and brain plasticity and enhanced functional recovery after stroke (Chen et al., 2005b). Interestingly, tissue plasminogen activator (tPA)-mediated aggravation of focal cerebral ischemia was reversed by rosuvastatin, but this effect appeared to be independent of eNOS (Kilic et al., 2005a). Atorvastatin also reduced exogenous tPA-induced expression of CEC genes that mediated thrombosis and increased BBB permeability. Inhibition of these genes including MMP-2 and -9 could contribute to the beneficial effects of statins in thrombolytic therapies of acute stroke (Liu et al., 2005). Postischemic delivery of rosuvastatin protected the brain against focal cerebral ischemia in mice by inhibiting ERK phosphorylation, caspase-3 activation, and iNOS while sparing eNOS (Kilic et al., 2005b). In an in vitro experimental paradigm, pretreatment with pravastatin led to activation of HIF-1. This was accompanied by induction of VEGF in mouse CECs, attenuating cytotoxicity mediated by ceramide, a pro-apoptotic lipid messenger in the ischemic brain (Chen et al., 2005c). These results together reveal multiple molecular mechanisms underlying the statin-mediated protective effects in cerebral ischemia–reperfusion (*Figure 10-3*).

7.2 Preconditioning

Neuroprotection against cerebral ischemia can be realized if the brain is preexposed to a sublethal ischemia known as IPC. Possible endothelial mechanisms that underlie neuroprotection conferred by IPC have been reviewed earlier. IPC attenuated postischemic BBB breakdown and vasogenic brain edema in a rat MCAO model (Masada et al., 2001). NO produced by eNOS appeared to mediate this protective effect (Gidday et al., 1999). This contention is supported by the finding that IPC conferred no protective effects in either eNOS or nNOS knockout mice, suggesting that NO may contribute to IPC-dependent protection (Atochin

Figure 10-3

Effects of statins on ischemic vascular injury. Statins, 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors, may exert multiple protective actions independent of its cholesterol-lowering effects. These include enhancement of intracerebral circulation via induction of eNOS, activation of VEGF and its cognate receptor for enhanced angiogenesis, and expression of BDNF for neurotrophic effects. Statins can also suppress expression of iNOS and MMP with preservation of BBB integrity. Furthermore, statins inhibit caspase-3 and platelet activation to confer antiapoptotic and anti-inflammatory actions, respectively. When coupled with tPA, statins inhibit tPA-induced thrombosis after cerebral ischemia



et al., 2003). In addition to IPC, bacterial endotoxin LPS also had neuroprotective effects against ischemia if administrated prior to MCAO in rats (Ahmed et al., 2000). Significant preservation of intracerebral blood flows in the peri-infarct area with sustained elevation of eNOS expression could contribute to LPS-induced tolerance to ischemia (Furuya et al., 2005). Despite these studies pointing to the critical importance of eNOS in mediating the IPC or LPS-induced ischemic tolerance, other studies reported different results. In one Study, endothelium-derived NO played a role in the brain tolerance induced by LPS while 70-kDa heat-shock protein was involved in the protection conferred by IPC but not LPS (Puisieux et al., 2000). In another study, prenatal hypoxia-induced ischemic tolerance in newborn rats appeared to involve iNOS rather than eNOS or nNOS (Zhao and Zuo, 2005).

7.3 Prostaglandins

Prostacyclin (PGI_2), a potent vasodilator and inhibitor of platelet aggregation and leukocyte activation, may be crucial in vascular diseases such as stroke. Prostacyclin synthase (PGIS), the key enzyme for PGI_2 synthesis, is mainly localized in endothelial cells in large as well as small vessels. PGIS expression was induced after cerebral ischemia in neurons in the ischemic penumbra. Overexpression of PGIS by adenoviral gene transfer 72 h, but not immediately, before ischemic insult substantially reduced infarct volumes by enhancing PGI_2 synthesis to create a favorable PGI_2/thromboxane A_2 ratio (Fang et al., 2006). In another study, adenoviral bicistronic COX-1/PGIS and COX-1 gene transfer also reduced cerebral infarct volumes by augmenting synthesis of protective prostaglandins when gene transfer was given as late as 5 h after ischemia. Adv-COX-1/PGIS infusion selectively augmented prostacyclin levels, with concurrent reduction in contents of other eicosanoids and leukotrienes in the ischemic cortex. Adv-COX-1 had similar effects on changes in the eicosanoid profile and neuroprotection as those achieved by Adv-COX-1/PGIS. The neuroprotective effects of Adv-COX-1 could be blocked by a selective COX-1 inhibitor (Lin et al., 2002). As mentioned earlier, PGI_2 inhibited NADPH oxidase activity to reduce ROS generation under hypoxia. Another protective mechanism of PGI_2 involved its stabilization of HIF-1α in endothelial cells (Chang et al., 2005). Prostaglandin (PG) D_2 (PGD_2) and its metabolite, 15-deoxy-Δ-(12,14) PGJ_2 (15d-PGJ_2), are also neuroprotective against ischemia–reperfusion injury. Intraventricular infusion of 15d-PGJ_2 inhibited neuronal apoptosis and necrosis and reduced infarct volumes. These 15d-PGJ_2 effects were blocked by a peroxisome proliferators-activated receptor-gamma (PPARγ) inhibitor. Adv-COX-1 also increased cortical

15d-PGJ_2 levels in rats in a manner that was correlated with reduction in infarct volumes and increase in PPARγ and HO-1 activity. Infusion of rosiglitazone, a PPARγ agonist, had a similar effect (Lin et al., 2006). In another study, rosiglitazone or 15d-PGJ_2 reduced infarct volumes and improved neurological scores in an MCAO model in rats. These two compounds also shared similar actions in ischemia-induced expression of iNOS and MMP-9 via the NF-κB signaling pathway (Pereira et al., 2006). Altogether, these recent findings support the contention that modulation of prostaglandin metabolism predominantly shown in CECs is an attractive strategy to protect the brain against ischemic injury.

7.4 Other Strategies

Preservation of endothelial functions with low levels of NO and inhibition of excessive production of NO from iNOS is a potential therapeutic approach for acute stroke. *S*-Nitrosoglutathione (GSNO) provided neuroprotection in a rat model of focal cerebral ischemia by inhibiting NF-κB transactivation of iNOS expression, tumor necrosis factor-α, and interleukin-1β (Xu et al., 1997; Khan et al., 2005). Corticosteroids increased cerebral blood flow and exerted stroke protection by rapid activation of eNOS that did not involve the conventional transcriptional regulation mechanism via the glucocorticoid receptor (GR). Instead, GR rapidly activated eNOS through the PI3K/Akt pathway via an extranuclear mechanism to exert an acute neuroprotective action that augmented cerebral blood flow (Limbourg et al., 2002).

8 Summary

In the past two decades, studies on CEC responses to ischemia–reperfusion insult have contributed to the understanding that CEC dysfunction and death play major roles in the secondary injury following cerebral ischemia–reperfusion and in the pathogenesis of vasogenic brain edema and hemorrhagic transformation. The secondary events affecting CECs are major determinants of stroke outcomes, including mortality. Studies exclusively on CECs in vitro or vascular injuries in the ischemic brain in animal stroke models in vivo have identified a number of mediators including ROS, cytokines, enzymes, and others that activate selective injury or death-signaling pathways to cause CEC dysfunction and death. These advances have led to the delineation of molecular and cellular mechanisms that could be modulated to minimize ischemic brain injury and to improve functional recovery after stroke by enhancing CEC viability and function. Effective therapeutic interventions are likely to be derived based on better understanding of CEC responses to ischemia–reperfusion.

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11 Gender, Sex Steroids, and Cerebral Ischemic Pathobiology

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Abstract: Biological sex is an important genetic determinant of outcome from cerebral ischemia and clinical stroke. Emerging data suggest that sex, as well as reproductive steroids, shapes ischemic cell death in brain. Female sex steroids, the estrogens and progesterone, provide robust neuroprotection in a variety of experimental settings and strongly contribute to sex-specific responses to ischemia. The purpose of this chapter is: (1) to review the importance of biological sex to ischemic outcome and mechanisms of brain injury, (2) to evaluate the role of female sex steroids as endogenous or exogenous ischemic neuroprotectants, and (3) to review most likely mechanisms by which female sex steroids act to interrupt ischemic cell death pathways.

List of Abbreviations: AP1, activator protein 1; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Ca^{2+}, calcium; cAMP, cyclic adenosine monophosphate; CREB, cAMP-responsive element-binding protein; E14, embryonic day 14; E2, estradiol; ER, estrogen receptors; αERKO, estrogen receptor deficient knockout mice subtype α; βERKO, estrogen receptor deficient knockout mice subtype β; ERE, estrogen-response elements; GAD, glutamic acid decarboxylase; i.p., intraperitoneal; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; MAPK, mitogen-activated protein kinase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NMDA, *N*-methyl-*D* aspartate; NF-κB, nuclear factor-kappa B; OVX, ovariectomized; ERK1 and ERK2, p42/p44 extracellular signal-regulated kinases 1 and 2; ONOO, peroxynitrate; PARP-1, poly-ADP ribose polymerase; PR, progesterone receptor; PKC, protein kinase C; RSF, reproductively senescent female; SHRSP, spontaneously hypertensive stroke prone; SD, Sprague-Dawley; TBI, traumatic brain injury

1 Introduction

Stroke or brain attack is a sexually dimorphic disease. Women enjoy protection from stroke relative to men, and this sex difference in risk-of-stroke persists well beyond the menopause. In animal models, stroke sensitivity as measured by tissue damage resulting from an ischemic event is also sex specific. In part, these differences are due to cellular actions of female sex steroids: the principal mammalian estrogen, 17β-estradiol and the progesterone family (Hurn and Macrae, 2000; Hurn and Brass, 2003; Murphy et al., 2003, 2004). The purpose of this chapter is: (1) to review the importance of biological sex to ischemic outcome and mechanisms of brain injury, (2) to evaluate the role of female sex steroids as endogenous or exogenous ischemic neuroprotectants, and (3) to review most likely mechanisms by which female sex steroids act to interrupt ischemic cell death pathways. New data suggest that androgens also play a role in ischemic outcome, however this emerging area of research will not be reviewed here.

2 Biological Sex Differences in Response to CNS Injury

2.1 Clinical Stroke Incidence and Outcome in Men and Women

It is now well recognized that there are biological sex-linked patterns of cerebrovascular disease and stroke. Overall incidence of stroke is higher in men compared to women in all countries and across ethnic backgrounds (Sudlow and Warlow, 1997). This sexually dimorphic epidemiology remains apparent until ages well beyond the menopausal years (Giroud et al., 1991; Sacco et al., 1998). For example, in the Northern Manhattan Stroke Study, stroke rates in women do not equalize to those of men until beyond 75 years of age (Sacco et al., 1998). Nevertheless, stroke risk increases with age in both sexes, and there is some evidence that outcome from an ischemic event is worse in aged women than in their male counterparts (Bousser, 1999). Knowledge of mechanisms of ischemic cell death and neuroprotection is important for both sexes. However, as discussed below, tantalizing new evidence suggests that these mechanisms may not be identical in male and female animals.

2.2 Sex Differences in Ischemic Outcomes: Animal Models

Early evidence in female versus male spontaneously hypertensive, genetically stroke prone (SHRSP) rats emphasized that the male phenotype is "ischemia-sensitive." In a study of 2,000 animals, Yamori et al. (1976) showed that life expectancy is longer in the female SHRSP with delayed development of cerebral hemorrhage and vascular lesions. In adult animals, experimental outcome from adult brain injury is clearly sex linked. Female rats and mice of various inbred and outbred strains experience smaller tissue damage for an equivalent insult from focal or global cerebral ischemia (Hall et al., 1991; Alkayed et al., 1998; Carswell et al., 1999; Alkayed et al., 2000; McCullough et al., 2003) and improved functional outcome (Li et al., 2004). Similarly, male animals sustain greater injury than do age-matched females after tramatic brain injury (Bramlett and Dietrich, 2001). We have explored complicated rodent models with genetic risk factors associated with human stroke, e.g., insulin-dependent genetic diabetes (Toung et al., 2000), non-insulin-dependent diabetes (Vannucci et al., 2001), and hypertension (Alkayed et al., 1998). In each genetic strain and despite deleterious complications from diabetes or hypertension, females are less sensitive to cerebral ischemia than are males. These data suggest that male animals, unlike females, must cope with a basal "ischemia-sensitive" phenotype.

2.3 Sex-Specific Cultures: Hormone-Independent Cell Death or Survival

Data from cell cultures in which background sex steroids are removed support the concept that cell death mechanisms can be sex specific. Some molecular pathways of cell death or survival diverge, depending on the genetic sex of the tissue (defined as female XX or male XY). Sex alters cell fate. For example, cultured female dopaminergic neurons (embryonic day 14, E14) tolerate exposure to toxic dopamine concentrations at the LD50 level and survive twofold relative to male cells (Lieb et al., 1995). Similarly, female neurons (E19) from cortical plate or ventricular zone have greater longevity in culture than do male cells and differentially express higher levels of phosphorylated kinases such as Akt (Zhang et al., 2003). Sensitivity to glutamate, peroxynitrate (ONOO), and staurosporine in neuronal culture (E17) is sex specific, with male neurons being more susceptible to glutamate and ONOO than females. In contrast, response to oxidants such as H_2O_2 is gender neutral (Du et al., 2004). These observations are not limited to neurons. Cell death resulting from oxygen–glucose deprivation is less in female versus male astrocytes cultured from rat or mouse at postnatal day 3 (Liu et al., 2004).

2.4 Sex-Specific Cell Death Mechanism: NO Toxicity and Activation of Poly-ADP Ribose Polymerase

Data from genetically engineered mice also suggest that molecular mechanisms of cell injury are not necessarily identical in male and female brain. When both sexes are studied, ischemic outcome in transgenic mice can be overtly gender dependent, even when the gene of interest is not linked to sexual development, e.g., inducible or neuronal nitric oxide synthase (Loihl et al., 1999; Sampei et al., 2000b; McCullough et al., 2004). For example, it is well accepted that the neuronal nitric oxide synthase (nNOS) plays an important role in initiating ischemic cell death. In large part, nitric oxide cytotoxicity involves its rapid reaction with superoxide anion, resulting in peroxynitrite formation and protein nitration. Genetic deletion or pharmacological inhibition of nNOS is neuroprotective in male animals, presumably by reduction of available nitric oxide (NO). However, loss of nNOS in female knockouts or with enzyme inhibition paradoxically increases histological infarction after middle cerebral artery (MCA) occlusion (McCullough et al., 2004). Activation of poly-ADP ribose polymerase (PARP-1) is a critical component of neuronal cell death after exitotoxic or ischemic insults in male mice or mixed cell culture unseparated by genetic sex (Eliasson et al., 1997; Goto et al., 2002). Surprisingly, loss of PARP-1 activity in female knockouts or after PARP inhibition hugely exacerbates ischemic damage after MCA occlusion (McCullough et al., 2004). While it is not clear

how these cell death pathways diverge in the male and female, these data suggest that sex matters at the molecular level in ischemic brain injury.

3 Estrogen

Estrogen has been extensively studied in the last decade, in part, because of its potential role in protecting premenopausal women from cardiovascular disease and stroke (Hurn and Macrae, 2000; Hurn and Brass, 2003). Mammalian estrogens are naturally synthesized from testosterone by aromatization, a process that occurs not only in gonads, placenta, and fat but also in brain (Naftolin, 1994; Azcoitia et al., 2003). Estradiol (E2) is the most potent and abundant of the three major human estrogens: estrone, estriol, and E2. Accordingly, it has been studied comprehensively as a model hormone. There are two stereoisomers of E2, 17α and 17β, but only 17β-estradiol binds effectively to estrogen receptors (ER).

3.1 Estradiol Signaling

Classical E2 signaling is via binding to ERs, followed by transcriptional regulation of target genes. Recent studies have also identified nontranscriptional rapid signaling effects of E2, mainly phosphorylation and activation of proteinases and kinases (Kelly et al., 2003; Maggi et al., 2004), which affect ion currents (Lagrange et al., 1996; Mermelstein et al., 1996), sensitivity of neurotransmitter receptors (Weaver Jr et al., 1997; Disshon et al., 1998), and transcriptional regulation of genes that do not carry classic estrogen-response elements (ERE) (Webb et al., 1999). These effects have, at least in part, been attributed to E2 binding to a putative membrane-bound ER (Toran-Allerand et al., 2002).

3.1.1 ER-Mediated Gene Transcription

Many physiological effects of E2 are mediated through binding to ER proteins. Two receptor subtypes, termed ER-α and ER-β, have been identified. The receptors form homo- and heterodimers upon activation (Cowley et al., 1997), and the resulting complexes then bind to ERE within the promoter of target genes and alter rate of gene transcription. Recent data suggest that ER complexes also interact with other transcription factors, e.g., nuclear factor-kappa B (NF-κB) and activator protein 1 (AP1) (Paech et al., 1997; McKay and Cidlowski, 1999). In addition, E2-induced transcription can be influenced by cofactors and coactivators, e.g., androgen receptor associated protein ARA-70 (Yeh et al., 1998). EREs have been identified in genes implicated in normal brain function and pathology, e.g., choline acetyltransferase (Miller et al., 1999), α1a-adrenergic receptor (Lee et al., 1998), oxytocin (Adan et al., 1993) and its receptor (Bale and Dorsa, 1997), preproenkephalin (Zhu and Pfaff, 1995), somatostatin (Xu et al., 1998), galanin (Kofler et al., 1995), glial fibrillary acidic protein (Stone et al., 1998), brain-derived neurotrophic factor (Sohrabji et al., 1995), transforming growth factor-α (El Ashry et al., 1996), cyclin D1 (Sabbah et al., 1999), and bcl-2 (Teixeira et al., 1995).

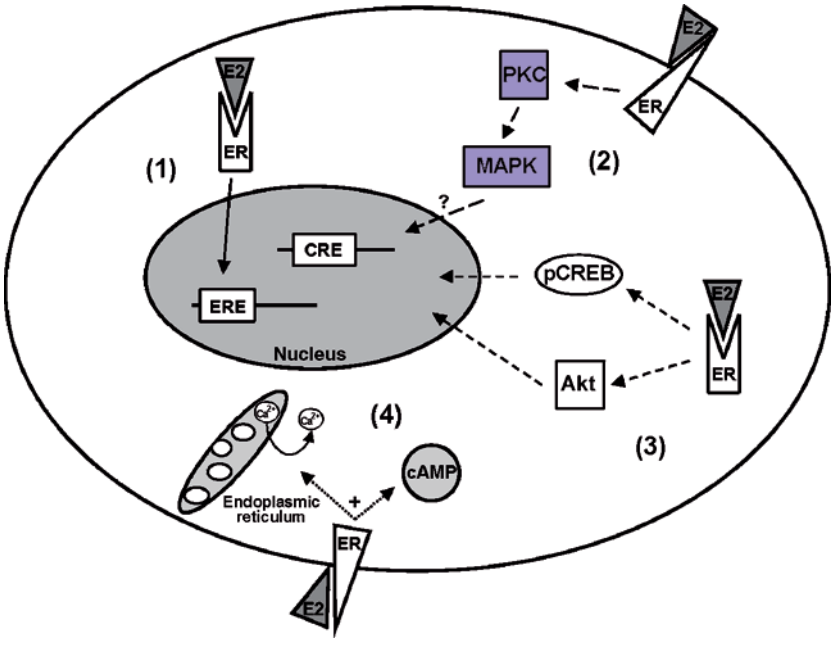
3.1.2 ER-Mediated Rapid Signaling

Recent studies have shown that E2 also elicits rapid intracellular signaling, independent of protein synthesis. These data suggest an additional action on yet unidentified membrane or cytoplasmic sites or with a putative membrane-bound ER (Toran-Allerand et al., 1999, 2002; Kelly et al., 2003). Rapid actions include modification of protein phosphorylation and levels of intracellular signaling molecules such as cyclic adenosine monophosphate (cAMP) (Minami et al., 1990) or calcium (Beyer and Raab, 1998). Activation of protein kinase C (PKC) (Ansonoff and Etgen, 1998), phosphorylation and activation of cAMP-responsive element-binding protein (CREB) (Zhou et al., 1996; Watters and Dorsa, 1998) and Akt (Singer et al., 1999),

and stimulation of the mitogen-activated protein kinase (MAPK) pathway (Singh et al., 1999) are additional important downstream factors involved in rapid signaling. Many of these effects may be caused by an interaction of E2 and ER with membrane and receptor-bound G-proteins (Mermelstein et al., 1996) (*Figure 11-1*).

Figure 11-1

Basic estrogen signaling pathways relevant for neuroprotection. (1) Classical gene transcription: 17β-estradiol (E2) binds to cytoplasmic estrogen receptor (ER) and increases gene transcription via estrogen response element (ERE). (2) Putative membrane regulated signaling: E2 activates protein kinase C (PKC) and mitogen activated protein kinase (MAPK) pathway after binding to a membrane-bound receptor. The current hypothesis is that this induces transcription. (3) Initiation of transcription through non-ERE transcription factors: E2 phosphorylates and activates Akt and cAMP-responsive element binding protein (CREB). (4) Intracellular increase in second messengers: E2 raises levels of second messengers cyclic adenosin monophosphate (cAMP) and calcium (Ca^{2+}). Current hypothesis is that this is a nontranscriptional mechanism. Any one of these pathways, or their combination, can eventually increase cell and tissue resistance toward ischemia



3.1.3 Non-receptor-Mediated Mechanisms

In vitro studies suggest that 17β-E2 or 17α-E2 can protect cells against a variety of stressful stimuli in ER deficient cell lines or in the presence of ER antagonists such as tamoxifen (Regan and Guo, 1997; Culmsee et al., 1999). Common stressors studied include hypoxia and glutamate or AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) toxicity. The data suggest that E2 has non-receptor-mediated effects in addition to the mechanisms outlined above. At least in part, these effects may be due to the hormone's antioxidative potential. Supraphysiologic concentrations of E2 (μM concentrations) can prevent intracellular peroxide accumulation and lipid peroxidation by inactivation of free radicals that could otherwise oxidize lipoproteins (Keller et al., 1997; Vedder et al., 1999). Both 17α-E2 and 17β-E2 possess antioxidant properties, emphasizing that the ER does not play a role (Behl and Manthey, 2000). E2 may also interact directly with neurotransmitter receptors, e.g., *N*-methyl-D-aspartate (NMDA) receptors to influence

neurotransmitter uptake or ion fluxes (Weaver et al., 1997). However, very high concentrations are required for potent antioxidation, so this mechanism may not be dominant under physiological conditions.

3.1.4 Dose Specificity

Experimental studies have used a large array of estrogen formulations and doses, not exclusively E2. Physiologic plasma levels of 17β-E2 range between 10 and 30 pg/ml in rat, for example, reaching peak levels of up to 140 pg/ml over the course of the estrous cycle (Butcher et al., 1974; Nequin et al., 1979). Chronic treatment of male or ovariectomized female animals with E2 doses that produce physiological plasma levels produces neuroprotection in a variety of animal models (Toung et al., 1998; Rusa et al., 1999; Alkayed et al., 2000). However, acute treatment strategies or treatment with supraphysiological doses (in some cases one or two orders of magnitude above the physiological range) do not always provide protection in vivo (Dubal et al., 1998; Rusa et al., 1999). The optimal dose or plasma level required for neuroprotection can differ, depending on species, injury model, duration, and timing of exposure. The requirement for physiological versus pharmacological concentrations/doses provides clues as to the dominant protective mechanism at work. For example, physiological levels are thought to act predominantly via ER signaling, whereas direct antioxidant actions would be predicted to result from supraphysiological hormone doses.

3.1.5 ERs in Brain

Not surprisingly, the highest levels of ER expression are in brain regions associated with reproductive function such as hypothalamic nuclei (Pfaff and Keiner, 1973). However, both ER-α and ER-β are also expressed at lower levels in brain regions relevant to cerebral ischemia, e.g., neocortex and hippocampus (Shughrue and Merchenthaler, 2000). At present, ER expression has been identified in a wide range of cell types under normal conditions. These include the cerebral vasculature, neurons, astrocytes, microglia, and oligodendrocytes (Azcoitia et al., 1999; Gudino-Cabrera and Nieto-Sampedro, 1999; Mor et al., 1999). Although sex differences have not been widely studied, ER mRNA is expressed to a higher extent in brains of adult females as compared to males (Shughrue et al., 1992). The importance of ERs, and of the specific ER subtype, for neuroprotective signaling remains unclear. Both ER-α and -β are differentially expressed in brain, although with some overlap (Li et al., 1997; Greco et al., 2001), suggesting that ER-mediated mechanisms of neuroprotection are possible. Current data using ER deficient knockout mice subtype α (αERKO) or subtype β (βERKO) do not fully clarify this issue. Enhanced damage after reversible MCA occlusion is not observed in gonadally intact αERKO mice of either sex relative to their respective wild type controls (Sampei et al., 2000a). In a permanent occlusion model, E2 replacement in ovariectomized αERKO females, but not βERKO females, is ineffective, suggesting that E2 signals through ER-α to reduce stroke damage (Dubal et al., 2001). Rapid E2 signaling involving phosphorylation of CREB and MAPK can be elicited in the absence of either one of the classical receptors, but not in double-knockouts (Abraham et al., 2004). The importance of ER-controlled mechanisms to neuroprotection requires further investigation.

3.2 Estradiol as a Neuroprotectant

E2's physiological signaling mechanisms have been well described, however, less is known in the context of cerebral ischemia. A large body of evidence demonstrates that the steroid protects cultured cells and brain tissue from ischemic damage. Details of these studies are summarized in *Tables 11-1* and *11-2* and discussed below.

Table 11-1
Representative in vitro studies of estradiol as a neuroprotectant

Tissue or cell type	Injury or stress	Key effects	Source
Cortical neurons	Glutamate (0.1 M)	Reduces cell death	Singer et al. (1996)
Hippocampal neurons	Glutamate (30 μM)	Reduces cell death	Weaver et al. (1997)
Rat mesencephalic neurons	Oxidative stress glutamate (1 mM), superoxide anion/H_2O_2	Reduces cell death, independent of ER, nongenomic mechanism	Sawada et al. (1998)
Dorsal root ganglion neurons	Growth-factor withdrawal	Increases cell survival; ER-dependent upregulation of bcl-*x*	Patrone et al. (1999)
Rat cortical neurons	Anoxia	Reduces cell death, ER independent	Zaulyanov et al. (1999)
Nigral neurons	Bleomycin sulfate or buthionine sulfoximine	Reduces apoptosis; ER dependent; independent of caspase-3 and JNK activation	Sawada et al. (2000)
Mouse dopaminergic neurons		Stimulates neurite outgrowth via CREB phosphorylation; ER independent	Beyer and Karolczak (2000)
Human neurons	Toxins: methamphetamine, cocaine and HIV protein	Reduces cell death; ER dependent	Turchan et al. (2001)
Purified neurons with ER-α versus astrocyte/neuronal coculture	Multiple insults	Protection only present in coculture system	Dhandapani and Brann (2003)
HT-22 cells	Iron-induced lipid peroxidation	Reduces peroxidation and increases cell survival	Vedder et al. (1999)
Murine hippocampal HT-22 cells	Oxidative stress	17β-estradiol and enantiomer ENT-E(2) reduce cell death	Green et al. (2001)
Neuroblastoma SK-ER3 cells		Increases expression of antiapoptotic nip-2; reduces apoptotic cell death; ER dependent	Meda et al. (2000)
PC-12 cells	Oxidized low density lipoprotein	17β, but not 17α-estradiol, reduces death	Berco and Bhavnani (2001)
Hypothalamic cell line		Promotes cell survival, ER-α required; Fas and FasL signaling required	Nilsen et al. (2000)
Slice, mouse hippocampus	Deafferentation of the entorhinal cortex	ER-dependent increase in sprouting of mossy fibers	Teter et al. (1999)
Slice, rat cortex	Kainic acid or potassium cyanide	Reduces cell death only at physiological levels (1–30 nM); protection lost at higher doses	Wilson et al. (2000)
Slice, rat hippocampus	Glutamate (1 mM)	Increased expression of CA3 NMDA receptor, associated with increased cell death	Sato et al. (2002)

Table 11-2
Representative in vivo studies of estradiol as a neuroprotectant

Ischemia model	Animals studied	Treatment	Follow-up	Key effects	Source
Focal (middle cerebral artery occlusion, MCAO), transient	Ovariectomized (OVX) female Sprague-Dawley (SD) rats	1-mg/kg 17β-E2 at reperfusion	23 h	Reduced infarct size	Zhang et al. (1998)
	OVX SD	E2 implant 24 h before MCAO	6 h, 24 h, 1 week	Reduced infarct size; reduced mortality	Simpkins et al. (1997)
	OVX SD	100-μg/kg E2 s.c., 2 h before MCAO	24 h	Reduced infarct size	Fan et al. (2003)
	Male Wistar	1-mg/kg Premarin i.v. at reperfusion	22 h	Reduced infarct size	McCullough et al. (2001)
	Male Wistar, intact or castrated	E2 25- or100-μg implants 1 week, or Premarin 1 mg/kg i.v. 30 min before MCAO	22 h	Reduced infarct size	Toung et al. (1998)
	OVX Wistar	E2 25- or 100-μg implants 1 week, or Premarin 1 mg/kg i.v. at MCAO	22 h	Reduced infarct size with chronic, no effect with acute treatment	Rusa et al. (1999)
	OVX Wistar	0.1- or 1-mg/kg E2 i.v. 30 min before MCAO or 20- or 200 μg s.c. for 1 week	72 h	No effect	Vergouwen et al. (2000)
	Reproductively senescent female Wistar rats	25-μg E2 implant 1 week before MCAO	22 h	Reduced infarct size	Alkayed et al. (2000)
	Male diabetic rats	25-μg E2 implant 1 week before MCAO	22 h	Reduced infarct size	Toung et al. (2000)
	Male rats, intact or castrated	E2 implant 1 week before MCAO	23 h	Reduced infarct size	Hawk et al. (1998)
	Male mice and female OVX mice	E2 implants 180 μg/ml	2 week for histology	Improved motor-function; reduced atrophy in males only	Li et al. (2004)
Focal (MCAO), permanent	Male SD	E2 local 10/30 min before or 10-μg/kg E2 i.v. 30 min before/ 30 min after MCAO	4 h	Reduced infarct size	Saleh et al. (2001a, 2001b)

	Male mice	0.3–30 μg/kg E2 s.c. or i.p. 24 or 3 h before MCAO	2 day	Reduced infarct size	Culmsee et al. (1999)
	OVX SD, young and middle aged	1-mg/ml E2 implant 1 week before ischemia	24 h	Reduced infarct size	Wise and Dubal (2000)
	OVX SD, young or middle aged (3–4 or 9–12 months)	180- or 1000-μg/ml E2 implant 1 week before MCAO	24 h	Reduced infarct size	Dubal and Wise (2001)
	OVX spontaneously hypertensive rats	200-μg/kg/week estradiol valerate s.c., 3 week	3 day	Reduced infarct size	Fukuda et al. (2000)
	OVX SD	180- or 1000-μg/ml E2 implant 1 week before/at MCAO	24 h	Reduced infarct size with chronic, no effect with acute E2	Dubal et al. (1998)
Global (4 vessel occlusion)	OVX SD	E2 s.c. 24 h before ischemia	96 h	Reduced cell loss	He et al. (2002)
	OVX Wistar	25-μg E2 implant 1 week before ischemia	1 week	No protective effect	Harukuni et al. (2001)
Global (right carotid artery occlusion plus hypotension)	OVX SD	0.2-mg/kg/day E2 i.p., 2 week before ischemia	72 h	Reduced cell loss	Wang et al. (1999)
	OVX SD	0.1-, 0.5-, or 5-mg/kg/day E2 i.p., 2 week	72 h	Reduced cell loss at low doses	Pelligrino et al. (1998)
	Diabetic OVX SD	0.1-mg/kg/day E2 i.p., 1week	72 h	Increased cell loss, reduced function	Santizo et al. (2002)
Global (bilateral carotid artery occlusion)	OVX C57BL/6J mice	25-μg E2 s.c. 2 week before ischemia	72 h	Reduced cell loss	Horsburgh et al. (2002)
	OVX C57BL/6J mice	ER-α agonist PPT 2 mg/kg/day or ER-β agonist DPN (8 mg/kg/day) s.c., 1 week before ischemia	72 h	Reduced cell loss with ER-β, but not ER-α agonist	Carswell et al. (2004)
	Male gerbils	0.36-mg E2 s.c. 2 week before ischemia	7 day	Reduced cell loss	Jover et al. (2002)
	Male gerbils	3-, 10-, or 30-μg E2 i.c.v. or 4 mg/kg i.p. 1 h before ischemia	7 day	Reduced cell loss with higher doses	Chen et al. (1998, 2001)
	Male gerbils	0.05 or 0.025 μg/d E2 icv from 2 h before ischemia	7 d	Reduced cell loss	Sudo et al. (1997)

3.2.1 Effects of E2 In Vitro

Results from in vitro studies of experimental cell damage, e.g., excitotoxicity or oxidative stress, largely demonstrate protective effects. E2 is neurotrophic at physiologic concentrations (1–30 nM), stimulating neurite outgrowth and increasing survival of cultured neurons (Beyer and Karolczak, 2000; Meda et al., 2000). The presence of E2 in the media broadly protects primary cultures and neuronal cell lines against noxious stimuli, including hypoxia, glutamate or NMDA toxicity, oxidants, and many toxins. However, concentration–response relationships are complex and difficult to interpret among the studies. At supra-physiological concentrations, protection is ER independent and largely attributable to the hormone's antioxidative potency. Alternatively, ER-dependent regulation of apoptosis inhibiting genes is uncovered in the presence of physiologic E2 concentrations (Patrone et al., 1999; Meda et al., 2000). E2-mediated rapid signaling at physiologic concentrations contributes to altered gene expression (Beyer and Karolczak, 2000). It seems likely that E2 acts by different mechanisms in specific cell types, and accordingly, results in organotypic slices are less consistent than culture. In slice preparations, physiological E2 concentrations can reduce (Wilson et al., 2000) or exacerbate (Sato et al., 2002) cell death.

3.2.2 Effects of E2 In Vivo

Despite the complexity of E2's actions, most animal studies report profound neuroprotection. Chronic E2 replacement to physiologically relevant plasma levels reduces infarct size after transient and permanent focal ischemia in ovariectomized female rodents (see *Table 11-2* for representative studies). This effect is also seen in male animals and in complex disease strains like the SHRSP rat. Effects of high E2 doses are less clear, whether given as an acute treatment before or after focal cerebral ischemia (Dubal et al., 1998; Rusa et al., 1999). Similarly, studies of E2 in global cerebral ischemia demonstrate robust neuroprotection. Harukuni et al. (2001) observed surprisingly increased cell death in E2-treated, ovariectomized rats after four-vessel occlusion. However, E2 levels in treated animals were low in this study, suggesting that higher levels of circulating estrogen might be necessary for neuroprotection in this model. Effects of E2 on vascular behavior during the insult may be important as E2 can increase intraocclusion cerebral blood flow (He et al., 2002). However, there seems to be a ceiling for this effect (Pelligrino et al., 1998). Coexisting pathologic conditions, such as diabetes, in some settings can enhance undesirable actions of E2, exacerbating ischemic damage (Santizo et al., 2002).

To date, despite overall encouraging data, transfer of the positive experimental results to the clinic is hampered by study limitations: (1) experimental studies have mainly been performed in small rodents and are needed in higher order species; (2) most studies have focused on short-term histological outcomes and only few data are available for neurological/behavioral outcome parameters; (3) precise dose and duration data for any one model are limited.

3.2.3 Effects on Peri-Ischemic Cerebral Blood Flow

In vivo neuroprotection is, in part, related to E2's vadodilatory actions and the steroid's ability to promote optimal endothelial function (Mendelsohn and Karas, 1994; White et al., 1995). Under basal conditions, cerebral blood flow is higher in premenopausal women than in men or in older women (Davis et al., 1983; Shaw et al., 1984), suggesting a tonic role in the vasculature. Estrogen can also influence blood flow during ischemic stress and during reperfusion (Hurn et al., 1995; Alkayed et al., 1998; Pelligrino et al., 1998).

3.2.4 Effects on Postischemic Inflammation

Recent studies suggest that E2 exerts an anti-inflammatory effect after cerebral ischemia, by reducing the number of active microglia (Lei et al., 2003), by decreasing intravascular leukocyte adhesion and

migration into brain (Santizo et al., 2000), and by suppressing endothelial expression of adhesion molecules (Nathan et al., 1999; Mori et al., 2004). Moreover, E2 can suppress reactive gliosis and expression of the inflammation promoting transcription factor NF-κB after cerebral injury (Wen et al., 2004). Taken together, it appears that E2 can restrict cellular damage in the proinflammatory mileau of ischemia/reperfusion.

3.3 Exemplary Cellular Mechanisms of Protection

Significant work is now focused primarily on identification of genomic and nongenomic mechanisms that explain E2's neuroprotective properties in vitro and in vivo. One known genomic mechanism is through elaboration of antiapoptotic protooncogene bcl-2. Bcl-2 is well established for protective effects in brain injury of all types (for review, see Graham et al., 2000; Mattson et al., 2000). Under basal conditions and in the setting of ischemia, E2 enhances bcl-2 expression, presumably by transcriptional actions through one or more EREs with the bcl-2 promoter (Teixeira et al., 1995). E2 upregulates bcl-2 expression in several brain regions of cycling female rats (Garcia-Segura et al., 1998). E2 increases the expression of bcl-2 mRNA and protein in the peri-infarct region after transient MCA occlusion in rats, accompanied by a decrease in infarct size (Alkayed et al., 1998; Dubal et al., 1999; Alkayed et al., 2001). Moreover, bcl-2 overexpressing mice are protected from the exacerbation of cerebral ischemic injury ordinarily caused by ovariectomy in wild-type female mice (Alkayed et al., 2001).

Nongenomic signaling may also contribute significantly to E2-conferred resistance to insult and enhanced survival of neuronal cells. For example, rapid activation of the MAPK pathway has been implicated in several paradigms of cell death in vitro. E2 elicits rapid tyrosine phosphorylation and activation of MAP kinases ERK1 and ERK2 (p42/p44 extracellular signal-regulated kinases 1 and 2) in ER expressing cells. Subsequently, primary cortical or hippocampal neuronal cultures are protected from glutamate (Singer et al., 1999; Mize et al., 2003) as well as β-amyloid toxicity (Fitzpatrick et al., 2002). Additional studies are needed to completely understand the relevance of this vital signaling pathway in vivo.

Nonspecific mechanisms of protection are likely dominant at supraphysiologic levels of hormone exposure. High-dose E2 protects murine cortical cultures during oxidative stress from hemoglobin or sodium azide, and protection is independent of either the ER or of protein synthesis (Regan and Guo, 1997). Furthermore, E2 in large doses (0.3–30 mg/kg) can reduce infarct size in mice in an ER-independent fashion that is not blocked by tamoxifen (Culmsee et al., 1999). Protective effects of high-dose E2 may have clinical relevance for short-term treatment of patients after ischemic events who could benefit the plethora of receptor-mediated plus non-receptor-mediated hormonal effects. However, due to adverse effects of chronic exposure to large E2 doses, this approach has little relevance for hormone-replacement therapy.

3.4 Summary

Estrogen is a powerful neuroprotective agent in a variety of experimental models of cerebral ischemia. Protection is conveyed both by genomic and nongenomic mechanisms leading to increased cell survival after ischemia (*Figure 11-2*, *Table 11-3*). Whereas the experimental data are clear, the translation to humans is less certain at present. Despite repeated observational studies that estrogen/progestin therapy reduces cardiovascular disease and stroke risk in women, new prospective and randomized trials (e.g., Women's Health Initiative) do not support the long-term use of hormone replacement therapy. There are no trials to date that test the use of E2 or estrogen formulations in acute stroke therapy to ameliorate injury. We can extrapolate from the experimental data that the extent of protection E2 provides will be related to dose and cell specificity, as well as the presence of confounding diseases or ER polymorphisms. Future research should focus on unraveling the molecular mechanisms conveying estrogen's protective effects. In addition, the exact contribution of progesterone requires study if we are to understand hormone replacement therapy and stroke.

Figure 11-2
Estrogen's potential mechanisms of neuroprotection

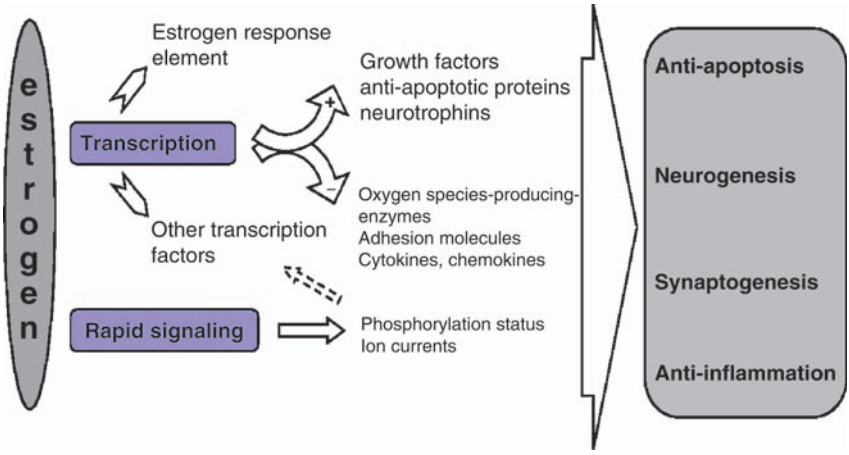


Table 11-3
Summary of potential estrogenic mechanisms of neuroprotection

Protective mechanism	References
Preservation of intraischemic blood flow	Alkayed et al. (2000)
Improvement of postischemic reperfusion	McCullough et al. (2001)
Antioxidant activity	Regan and Guo (1997)
Amelioration of excitotoxicity	Singer et al. (1999)
Upregulation of bcl-2 expression in injured area	Dubal et al. (1999), Alkayed et al. (2001)
Increase of neurite outgrowth, spine density, and cell viability in culture	Brinton et al. (1997), Murphy et al. (1998)
Increase of neurotrophic factors	Toran-Allerand (1996)
Reduction of leukocyte adhesion after transient global ischemia	Santizo et al. (2000)
Supression of microglial activation	Bruce-Keller et al. (2000)
Reduction of reactive gliosis	Garcia-Segura et al. (2001)
Increase of neuronal stem cell proliferation	Tanapat et al. (1999)

4 Progesterone and Neurosteroids

Progesterone, precursors, and metabolites can be generated locally from cholesterol in the brain as neurosteroids; formed from in situ metabolism of bloodborne precursors; or synthesized by the adrenal glands, placenta, or gonads. Pregnenolone is the immediate precursor of progesterone. Progesterone itself is partly transformed into 5α-pregnane-3, 20-dione (5α-dihydroprogesterone) and 3α-hydroxy-5α-pregnan-20-one (3α,5α-tetrahydroprogesterone or allopregnanolone). In the rat, plasma progesterone can range from basal levels of 2–18 ng/ml to approximately 120–130 ng/ml in pregnancy, with intermediate values of 40–90 ng/ml during late proestrus (Wiest, 1970; Butcher et al., 1974; Sutter-Dub et al., 1974; Nequin et al., 1979). The effects of these neurosteroids in brain range from neuroendocrine control of reproduction and sexual behavior, regulation of neurotrophicity, and modulation of such behaviors as anxiety, stress, sleep, and memory.

Neurosteroids exert their actions on target brain cells through genomic and nongenomic actions. Progesterone classically binds to a selective intracellular receptor. There are two forms of the progesterone

receptor (PR), a large molecular form B and a smaller form A. Once activated, the PR interacts with a specific progesterone-response element within the promoter region of target genes. Other transcription factors, e.g., c-Jun or c-Fos, can also modulate PR transcriptional activity (Herrlich and Ponta, 1994). The PR appears in rat brain close to birth and increases during development in a region-specific manner (Kato et al., 1984). PR has been identified in neurons of the hypothalamus, cortex, amygdala, and cerebellum as well as in glial cells (Jung-Testas et al., 1984). Nongenomic mechanisms of action of progesterone and related neurosteroid metabolites in brain include: (1) intercalation into phospholipid bilayers; (2) binding to specific membrane receptors; (3) modulation of ion channels or ionic ATPase enzymes; (4) interactions with ligand-gated ion channels such as the $GABA_A$ and NMDA receptors; and (5) associations with G-protein–coupled receptors for neurotransmitters like the sigma or oxytocin receptors (for review, see El-Etr et al., 2000).

4.1 Progesterone and Outcome from Cerebral Ischemia

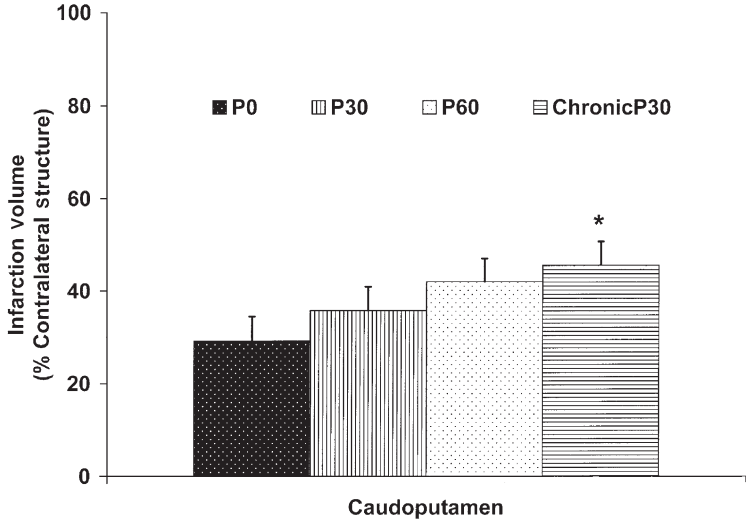
The effects of exogenous progesterone have been studied in rodent models and in higher order gyrencephalic animals such as cats. In transient focal and global cerebral ischemia models (*Table 11-4*), progesterone is neuroprotective in progesterone-deficient rodents [males, OVX females, and reproductively senescent females (RSF)]. Chronic progesterone administered before transient focal ischemia in OVX rats

Table 11-4
Exogenous progesterone in cerebral ischemia: animal studies

Gender/species	Effect on ischemic injury	Proposed mechanism	References
Male C57BL/6 mice	Reduced lesion volume, transient focal model	None tested	Gibson and Murphy (2004)
Male Sprague-Dawley rats	Reduced edema, permanent focal model	Not due to reduced blood–brain barrier permeability	Betz and Coester (1990a, 1990b)
Male Wistar rats	Reduced histological damage and improved neurological deficits, transient focal model	None tested	Jiang et al. (1996)
Male Wistar rats	Reduced infarction volume and improved functional outcome, transient focal model	None tested	Chen et al. (1999)
Male spontaneously hypertensive rats	Reduced lesion size and neurological deficits, transient focal model	None tested	Kumon et al. (2000)
Female OVX Wistar rats	Chronic preischemic treatment increased striatal infarction, transient focal model	None tested	Murphy et al. (2000)
Female OVX Wistar rats	Peri-ischemic treatment reduced cortical injury, transient focal model	Blood flow independent mechanism	Murphy et al. (2002)
RSF Wistar rats	Reduced cortical infarction, transient focal model	Blood flow independent mechanism	Alkayed et al. (2000)
RSF Wistar rats	No effect, transient focal model	None tested	Toung et al. (2004)
Female OVX cats	Reduced cell loss, transient global model	None tested	Gonzalez-Vidal et al. (1998), Cervantes et al. (2002)

Figure 11-3

Caudate-putamen infarction volume (% contralateral) at 22 h of reperfusion following 2-h middle cerebral artery (MCA) occlusion in ovariectomized female Wistar rats. Animals were treated with intraperitoneal injections: 0 (P0, $n = 14$), 30 (P30, $n = 14$), or 60 (P60, $n = 12$) mg/kg progesterone 30 min before ischemia or with 30-mg/kg progesterone (Chronic P30, $n = 16$) daily for 7–10 days before ischemia. *Significantly different from P0 ($p < 0.05$); values are means + SEM. Adapted from Murphy et al. (2000)



exacerbates caudoputamen infarction (*Figure 11-3*), but no mechanism of injury was tested in this study (Murphy et al., 2000). Because there are few studies of long-term progesterone exposure, the treatment duration requirement is not clear. Effective dose and duration may differ between sexes at various ages, suggesting that neuroprotective mechanisms are not necessarily identical in male and female animals throughout maturity. No studies are available to clarify effects of progesterone precursors and metabolites on ischemic outcome. However, in a rodent model of penetrating brain injury, pregnenolone and pregnenolone sulfate inhibits gliotic tissue formation (Garcia-Estrada et al., 1999). In traumatic brain injury models, allopregnanolone enhances behavioral recovery and decreases neuronal loss (Djebaili et al., 2004; He et al., 2004).

4.2 Comparison to Estrogen as a Neuroprotectant

Clinical and experimental inquiries have focused heavily on estrogen as a preventative agent in injured brain, but progesterone in some injury models has shown promise as a postinjury therapy in both males and females (for review, see Stein et al., 1999; Roof and Hall, 2000; Stein, 2001; Vink et al., 2001; Stein and Hoffman, 2003). Progesterone is a more attractive therapy for men, as chronic estrogen administration presents undesirable side effects and consequences. The use of estrogen alone (unopposed estrogen) in women is also problematic due to increased risk of uterine cancer (Grady et al., 1995). Therefore, estrogen is typically administered in combination with a progestin. Accordingly, progesterone may be more desirable in future clinical applications than estrogen.

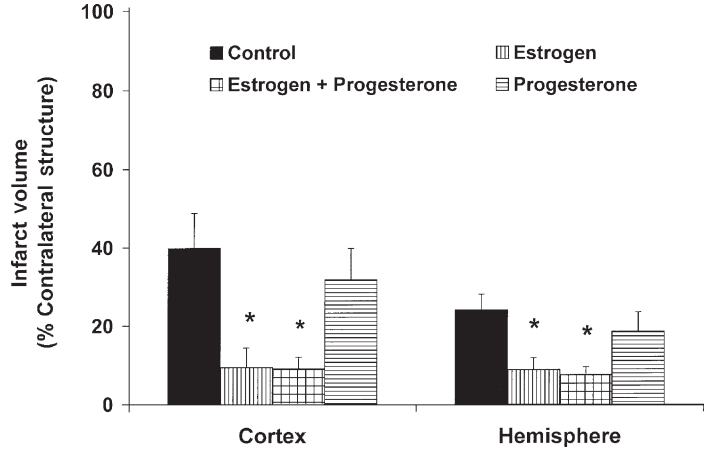
4.3 Combined Hormone Treatments and Outcome in Brain Injury Models

The emphasis on individual female sex steroids in experimental studies makes it difficult to evaluate interactive roles of estrogen and progestins. A single study has examined combined hormone administration

in cerebral ischemia (Toung et al., 2004). In this study, combined treatment in RSF rats was evaluated after reversible MCA occlusion. Subcutaneous estradiol initiated 7 or more days prior to occlusion combined with intraperitoneal (i.p.) progesterone injections at 30 min preischemia and through early reperfusion were compared to each steroid administered alone. Cortical, caudoputamen, and total infarct volumes were assessed by 2,3,5-triphenytetrazolium chloride staining and digital image analysis at 22 h reperfusion. Combined hormone administration and chronically administered 17β-estradiol alone reduced cortical and total brain ischemic injury (*Figure 11-4*) while progesterone alone did not reduce injury. These results suggest that in ischemic RSF rat brain, combined hormone administration reduces infarction volume and that progesterone does not attenuate estrogen's effect.

Figure 11-4

Cortical and total hemispheric infarction volume (% contralateral structure) at 22 h of reperfusion following 2-h MCA occlusion in reproductively senescent female (RSF) rats ($n = 10$/group). Animals were treated with no hormone (control), 25-μg estrogen subcutaneous implant (estrogen), 25-μg estrogen implant + 5 mg/kg progesterone i.p. (estrogen + progesterone), or 5-mg/kg progesterone i.p. (progesterone). All hormone implants were placed 7 days prior to MCA occlusion, and progesterone injections were given 30 min before occlusion, at initiation of reperfusion and at 6 h of reperfusion. *$p < 0.05$ compared to control; values are means + SEM. Adapted from Toung et al. (2004)



4.4 Mechanisms of Neuroprotection

While mechanisms of ischemic neuroprotection have not been evaluated, studies of traumatic brain injury (TBI) suggest potential protective mechanisms. For example, progesterone decreases cerebral edema (Roof et al., 1993; Roof et al., 1996; Roof et al., 1997; Galani et al., 2001; Wright et al., 2001; Grossman et al., 2004) in TBI models. Effects on cerebral edema may be partly mediated by progesterone's antilipid peroxidation capacity (Roof et al., 1997; Stein et al., 1999; Roof and Hall, 2000). Progesterone and allopregnanolone lower caspase-3 activity in TBI (Djebaili et al., 2004), suggesting these neurosteroids can decrease cell loss by decreasing caspase-3–dependent apoptotic mechanisms in this setting. Anti-inflammatory mechanisms are also important (Arvin et al., 1996; Garcia-Estrada et al., 1999; Grossman et al., 2004). Progesterone has direct effects on GABA neurotransmission by altering GABA conduction (Majewska, 1992), enhancing $GABA_A$ receptor numbers (Weiland and Orchinik, 1995), increasing $GABA_A$ agonist binding affinity (Jussofie et al., 1995) and number of binding sites (Juptner et al., 1991), and by enhancing glutamic acid decarboxylase (GAD) mRNA (Weiland, 1992; Grattan et al., 1996). Progesterone moderates neuronal responsivity to excitatory amino acids (Smith et al., 1987; Smith, 1991), thus ameliorating excitotoxic injury.

PRs have been implicated in vascular injury mechanisms. In carotid artery injury, OVX progesterone receptor knockout mice sustain more pronounced vascular injury than wild-type animals, independently of exogenous progesterone availability (Karas et al., 2001). The study suggested that PRs regulate vascular injury response in a complex manner and can augment or attenuate the degree of injury. In brain, progesterone's neuroprotection may be influenced by regional and temporal expression of PR subunits/subtypes. Finally, progesterone's actions in injured brain may be mediated, in part, by locally produced metabolites such as 5α-dihydroprogesterone and 3α,5α-tetrahydroprogesterone (allopregnenolone) (di Michele et al., 2000). In TBI in vivo (Djebaili et al., 2004; He et al., 2004) and excitotoxic injury in vitro (Lockhart et al., 2002; Ciriza et al., 2004), allopregnanolone provides neuroprotection similarly to progesterone.

4.5 Summary

Progesterone, its precursors, and its metabolites are produced endogenously within brain. These neurosteroids act by either altering specific gene transcription after binding to PRs or acting directly on the neuronal or glial plasma membrane. In the experimental setting, progesterone has an overall neuroprotective effect. However, the optimal dose and duration may differ between sexes at various ages, suggesting that steroid mechanisms are not necessarily identical in males and females as these animals mature. Little research has been done on combined estrogen and progesterone treatment in in vivo brain injury models, but one study suggests that combined hormone administration reduces infarcts and that progesterone does not attenuate estrogen's effect.

5 Conclusions and Emerging Hypotheses

Clinical and experimental evidence emphasize that many forms of brain injury are sexually dimorphic. Females are less vulnerable to cerebral ischemia, while the male phenotype is "ischemia sensitive." Novel evidence suggests that this dimorphism is present not only in the intact brain but also shapes the behavior of cultured XX versus XY cells. These differences may not be solely under hormonal control. Nevertheless, female sex steroids at physiological concentrations likely play a role in the female's endogenous neuroprotection. Both estrogen and progesterone can improve outcome from experimental brain injury. However, these pleiotrophic hormones have diverse cellular targets and the potential for competing mechanisms of action, leading to a "good, bad or null" profile. Future studies will harness the potential of these steroids as we demystify their mechanisms in brain protection.

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12 Mechanisms of Ischemic Cell Death in the Developing Brain

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List of Abbreviations: H–I, Hypoxia–ischemia; MRI, magnetic resonance imaging; CNS, central nervous system; P, postnatal day; PVL, periventricular leukomalacia; MCA, middle cerebral artery; CCA, common carotid artery; CBF, cerebral blood flow; CMRglc, cerebral metabolic rate of glucose; GLUT, glucose transporter; ATP, adenosine triphosphate; Na, sodium; K, potassium; Ca, calcium; EAA, excitatory amino acids; NMDA, N-methyl-D-aspartate; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate; nNOS, neuronal nitric oxide synthase; PSD, post-synaptic density protein; iNOS, inducible nitric oxide synthase; DNA, deoxyribonucleic acid; COX-2, cytochrome oxidase subunit 2; CuZn, copper zinc; SOD, superoxide dismutase; Gpx, glutathione peroxidase; Mn, manganese; H_2O_2, hydrogen peroxide; GSH, glutathione; BBB, blood brain barrier; IL-1β, interleukin-1 beta; TNFα, tumor necrosis factor alpha; CSF, cerebrospinal fluid; CD18Mab IB41, CD18 monoclonal antibody isolectin 41; ICAM-1, intracellular adhesion molecule; MCP-1, monocyte chemoattractant protein; MIP-1, macrophage inflammatory protein 1; AIF, apoptosis-inducing factor; APAF, apoptosis protease inducing factor; PARP, poly-adenosine diphosphate ribose polymerase; ROS, reactive oxygen species; DEVD-AMC, asp-glu-val-asp-aminomethyl coumarin; IAP, inhibitor of apoptosis; HIF, hypoxia inducible factor; NGF, nerve growth factor; BDNF, brain derived neurotrophic factor; BFGF, beta fibroblast growth factor; NT-3, neurotrophin 3; HSP, heat shock protein; VEGF, vascular endothelial growth factor; EPO, erythropoietin; MAPK, mitogen activated protein kinase; ERK, extracellular regulated protein kinase; JNK, jun kinase; OGD-, oxygen glucose deprivation; GFAP, glial fibrillary acidic protein; SVZ, subventricular zone

1 Introduction

While it has been known for a long time that hypoxia–ischemia (H–I) in preterm and term babies leads to neurologic disabilities, the recent advances in neonatal medicine and MRI (Ramaswamy et al., 2004) revealed a high incidence of neonatal stroke. Neonatal stroke occurs in about 1 out of 4,000 live births, rates comparable to those in the elderly, that affects central nervous system (CNS) development and is a major cause of cerebral palsy (Koelfen et al., 1995; deVeber et al., 2000; deVeber, 2002; Lynch et al., 2002). Although stroke in the neonatal period is increasingly studied, much of the groundwork for our understanding of pathophysiology comes from research in adult stroke models. Immaturity contributes to different mechanisms in cell death and injury progression after stroke (reviewed in Vexler and Ferriero, 2001; Vannucci and Hagberg, 2004), resulting in susceptibility to excitotoxic and oxidative injury in select populations and regions of the developing brain (Fullerton et al., 1998; Ikonomidou et al., 1999). In this chapter, we summarize knowledge from experimental studies on mechanisms of cell death following ischemic and H–I insults that contribute to injury propagation.

2 Animal Models of Preterm and Term Ischemic Brain Injury

2.1 Brief Overview of Large Animal Models

Rodents are the most frequently used animals in perinatal H–I research (reviewed in Ashwal and Pearce, 2001; Vexler and Ferriero, 2001; McQuillen and Ferriero, 2004; Vannucci and Hagberg, 2004), followed by piglets (LeBlanc et al., 1991; Adcock et al., 1996; Laptook et al., 1997; Roohey et al., 1997; Amess et al., 1998; Keller et al., 1998; Tooley et al., 2002), sheep (Klempt et al., 1993a; Roohey et al., 1997; Gunn et al., 1998; Gunn et al., 1999; Ohyu et al., 1999), rabbits (Derrick et al., 2004), and monkeys (Roohey et al., 1997; Naeye and Lin, 2001). Whole-brain and region-specific maturation varies greatly between different species, enabling investigators to study age-specific vulnerability of different regions and cell types to ischemic injury. While no single animal model is ideal for studying mechanisms of perinatal ischemia, some models are superior to others, depending upon the specific research question. The fetal sheep, newborn lamb, and piglet models are well suited for the study of acute and subacute metabolic and physiologic endpoints, whereas the rodent and primate models are used for long-term neurological and behavioral outcome experiments. Piglet and sheep models are the most suitable large animal models for studying effects of hypothermia on H–I injury (Laptook et al., 1997; Gunn et al., 1998, 1999; Tooley et al., 2002). Systemic

hypoxia/asphyxia with or without ischemia is the predominant method of producing experimental brain damage, followed by the use of excitotoxins and focally applied ischemia–reperfusion.

2.2 Small Animal Models

Several recently published reviews have summarized different small animal models relevant to ischemia in preterm and term babies (Ashwal and Pearce, 2001; Vexler and Ferriero, 2001; McQuillen and Ferriero, 2004; Vannucci and Hagberg, 2004). The most commonly utilized model of neonatal hypoxic encephalopathy at term gestation, a model of H–I (Rice et al., 1981; Vannucci, 1990), is used in postnatal (P) day P7–P10 rats and P7–P10 mice and requires a combination of unilateral carotid artery ligation and exposure to systemic hypoxia. The duration of a hypoxic episode sufficient to produce consistent unilateral injury varies from 30 min to 2.5 h depending on species and the background strain of the animals (Sheldon et al., 1998), and animals are followed through the juvenile period and young adulthood to determine short- and long-term consequences of H–I. Inducing H–I in younger animals (P1–P2) (Sheldon et al., 1996) results in selective white matter damage that resembles many features of periventricular leukomalacia (PVL), which is seen after hypoxia in preterm babies (reviewed in McQuillen and Ferriero, 2004). A model involving ibotenate injection into the brain serves as a model of excitotoxicity in term babies when used in P5–P7 rats and of white matter damage when used in P2–P3 pups (Gressens et al., 1996). The most recent addition to modeling ischemic injury in the immature brain is a model of transient middle cerebral artery (MCA) occlusion that enables determination of the role of reperfusion to injury in the immature rat brain without the confounding effect of systemic hypoxia. A model of MCA occlusion was originally developed in juvenile P14–P18 spontaneously hypertensive rats (Ashwal et al., 1995b) and later extended to normotensive P7 (Derugin et al., 1998) and P10 (Mu et al., 2003) rats. A model that employs a combination of permanent MCA occlusion and transient common carotid artery (CCA) occlusion in P7 rats (Renolleau et al., 1998) has also been developed.

3 Factors That Predispose the Immature Brain to Ischemic Injury

3.1 Cerebral Blood Flow and Metabolism

The presence of a "pressure passive" cerebral circulation has been demonstrated in preterm babies and in sick term infants, and the range of functional cerebrovascular autoregulation with increasing maturity has been shown in animals (Tuor and Grewal, 1994; Verma et al., 2000). Disruption of cerebral blood flow (CBF) results in the net movement of osmotically driven water from the extracellular to the intracellular space and occurrence of cytotoxic edema in neonatal rats shortly after H–I (Rumpel et al., 1997) (Dijkhuizen et al., 1998; Albensi et al., 1998; Tuor et al., 1998), focal transient ischemia (Derugin et al., 2000), and global ischemia (van der Toorn et al., 1996). Return to normoxia in the H–I model and blood recirculation in focal ischemic models do not necessarily compensate for disturbed cerebral metabolism (Malisza et al., 1999). Measurements of cerebral metabolic rate of glucose (CMRglc), measured as 2-deoxyglucose, in conjunction with CBF measurements (iodoantipyrine) in H–I models show that cortical regions that undergo ligation and systemic hypoxia increase CMRglc compared with the contralateral hemisphere 3–12 h after H–I, followed by a secondary phase with low CMRglc. In these regions, loss of microtubule-associated protein 2 and subsequent infarction (Gilland et al., 1998a) are observed. High CMRglc is unlikely to be caused by acidosis since intracellular pH becomes normal or alkaline shortly after reoxygenation (Yager et al., 1992).

3.2 Energy Metabolism

As in adults, glucose is the cerebral energy substrate in newborn. Unlike in adult brains, ketone bodies provide a substantial part of the cerebral energy substrate during the first two postnatal weeks and lactate can be used to a limited degree as an alternate energy source (Vannucci and Vannucci, 2000). Transition

from consuming ketones as brain substrates to glucose occurs gradually and, in rodents, is seen in the second postnatal week (Vannucci and Vannucci, 2000). The overall metabolic demands and activity of many energy-requiring pumps, like Na^+/K^+-ATPase, are lower in the neonatal than in the adult brain, rise gradually during the postnatal period, and hence parallel increases in levels of expression of glucose transporter (GLUT) proteins in the brain—the glial and endothelial transporter GLUT1 and the neuronal transporter GLUT3 (Vannucci et al., 1998). While the ability of the immature brain to use ketone bodies during hypoglycemia and H–I to partially substitute for glucose can provide some protection, glucose supplementation during but not after H–I is protective in the immature brain, whereas hypoglycemia is deleterious (Vannucci, 1990), which is opposite to effects seen in the adult ischemic brain. Glucose and ATP levels fall sharply during H–I, the immature brain becomes more dependent on anaerobic glycolysis, and cerebral glucose utilization increases substantially (Vannucci and Vannucci, 2000). Glucose levels increase twofold immediately upon return to normoxia (Vannucci et al., 1989), while incomplete restoration of ATP and phosphocreatine levels persist for the first few hours after H–I and lactate concentrations increase (Palmer et al., 1990; Malisza et al., 1999; Qiao et al., 2002). Restoration of post H–I ATP levels largely depends on mitochondrial function. Limitations in mitochondrial respiration, energy metabolism, and redox potential, as well as the capacity of brain mitochondria to handle increased intracellular Ca^{2+} become limiting factors that contribute to ischemic cell death acutely after injury (Yager et al., 1991, 1996; Gilland et al., 1998b; Keelan et al., 1999; Puka-Sundvall et al., 2000; Robertson et al., 2004). Structural and functional disturbances of mitochondria are largely responsible for apoptotic cell death in the neonatal brain (described in *Sect. 4.2* in this chapter).

3.3 Excitotoxicity and Selective Vulnerability of the Developing Brain

Neurotransmission plays an essential role in brain development and in learning. Glutamate, the major excitatory amino acid (EAA) in the mammalian brain, activates a variety of EAA receptors including N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole proprionate (AMPA) receptors (Nicoll and Malenka, 1995; Cain, 1997; Crepel et al., 1998). Under pathophysiological conditions associated with compromised energy metabolism, such as ischemia, when ATP-dependent processes fail leading to elevated levels of extracellular glutamate, EAA receptors become overactivated and mediate excitotoxicity. "Excitotoxicity" as a mechanism of neurodegeneration in general and the role of the NMDA receptor in particular have been well established in both acute and chronic neurological disorders and have become targets for adult antistroke therapies (Dirnagl et al., 1999).

The NMDA receptor is an important excitatory mechanism in both the immature and mature brain, and the same downstream signaling pathways exert intracellular signaling from the receptor in the immature and mature CNS. Expression and the relative composition of the four subunits of the NMDA receptor are unsynchronized during early postnatal development, resulting in deregulation of the receptor (Ikonomidou et al., 1989; McDonald and Johnston, 1990; McDonald et al., 1992; Ikonomidou et al., 1999; Bickler et al., 2004) and unwanted calcium entry into neurons. Further, an overall increased expression of the NMDA receptor during P5–P14 (Ikonomidou et al., 1989; McDonald and Johnston, 1990) also contributes to influx of Ca^{2+}, as well as Na^+ and water, leading to mitochondrial dysfunction and cell death after oxidative stress. Developmental differences in tyrosine phosphorylation of the NMDA receptor after H–I may also contribute to age-related changes in sensitivity to H–I injury (Gurd et al., 2002). These maturational differences may account for a "window of excitotoxic susceptibility" early postnatally (Ikonomidou et al., 1989), an idea consistent with findings that pharmacological antagonism of the NMDA receptor after stroke in adults can provide better protection (Dirnagl et al., 1999) than in the developing nervous system (Ford et al., 1989). Further, blockade of NMDA receptors early postnatally, even in the absence of H–I, triggers profound widespread apoptotic neurodegeneration in the developing rat brain (Ikonomidou et al., 1999).

Two mechanisms contribute to ischemia-induced increases in synaptic glutamate: an increased efflux from presynaptic nerve terminals and impaired reuptake by astrocytes. Glutamate efflux has both calcium-dependent (via voltage-dependent calcium channels) and -independent components (via a reversal of glutamate transporters). Removal of glutamate from the synaptic cleft is dependent on glutamate transporters

on astrocytes and is rapidly compromised after ischemia (Swanson et al., 1997; Chen and Swanson, 2003). The link between the NMDA receptor and neuronal nitric oxide synthase (nNOS) is provided by postsynaptic density (PSD)-93 and PSD-95 membrane-associated guanylate kinases. While strategies to inhibit activity of nNOS may reduce injury after H–I (Black et al., 1995; Ferriero et al., 1996; Muramatsu et al., 2000), the protective effects appear to be independent of PSD-93 (Jiang et al., 2003).

3.4 Nitric Oxide

Nitric oxide ($NO^{\bullet}$) plays an important role in the pathogenesis of neuronal injury during cerebral ischemia (reviewed in Beckman and Koppenol, 1996; Dirnagl et al., 1999; Lipton, 1999b). Depending on the cellular source and enzyme [endothelial (eNOS), neuronal (nNOS), inducible (iNOS)] isoform, $NO^{\bullet}$ generation can have paradoxical roles in the process of ischemic injury. While vasodilatation through induction of eNOS activity is beneficial, activation of nNOS and iNOS can be detrimental after neonatal H–I. Studies in immature rodents show a rapid biphasic rise in $NO^{\bullet}$ concentration following brain reoxygenation after H–I (Tsuji et al., 2000) and transient MCA occlusion in P7 (Coeroli et al., 1998) and SH P14–P18 (Ashwal et al., 1995a) rats. The expression of nNOS parallels that of the glutamate receptor during development and corresponds to regions of selective vulnerability to H–I (Black et al., 1995). A transient decrease in nNOS expression and NOS activity within 24 h after H–I in neonatal rat (Muramatsu et al., 2000) and mice (Ishida et al., 2001) is associated with a limited protection afforded by 7-nitroindazole, a relatively selective nNOS inhibitor. At the same time, destruction of neurons containing nNOS in the neonatal rat by quisqualate before H–I (Ferriero et al., 1995) or disruption of the nNOS gene (Ferriero et al., 1996) markedly lessens cortical injury.

Activation of iNOS, which is not energy dependent, predominantly occurs in reactive microglia/macrophages and reactive astrocytes and contributes to injury through the release of high levels of $NO^{\bullet}$(Beckman and Koppenol, 1996). $NO^{\bullet}$, in turn, can directly damage proteins and DNA as well as react with $O_2^{\bullet-}$ with the consequent formation of the potent oxidant and nitrating species peroxynitrite. Pretreatment with a nonspecific NOS inhibitor NG-nitro-L-arginine reduces infarct volume after H–I in P7 rats and transient MCA occlusion in juvenile rats (Ashwal et al., 1995a), and administration of a relatively selective iNOS and COX-2 inhibitor aminoguanidine has been shown to reduce injury after neonatal H–I (Tsuji et al., 2000) but not after neonatal focal transient ischemia (Dingman et al., 2004). Combined inhibition of nNOS and iNOS has been shown to reduce neuronal damage but not the inflammatory response following H–I in P12 rats (van den Tweel et al., 2002).

3.5 Oxidative Stress-Induced Injury to the Ischemic Neonatal Brain

The developing brain faces unique challenges from oxidative stress (Palmer, 1995; Fullerton et al., 1998; Sheldon et al., 2004) as maturational differences in the expression and activity of antioxidative enzymes impact the rate of utilization of reactive oxygen species (ROS), such as $O_2^{\bullet-}$ to $HO^{\bullet}$, and therefore the response of the newborn brain to injury. Dynamic changes occur in the activities of copper/zinc (CuZn)-superoxide dismutase (SOD), catalase, and glutathione peroxidase (Gpx) in the rat brain during the first 2 weeks of life. A decrease in catalase activity, an increase in CuZn-SOD (Aspberg and Tottmar, 1992; Buard et al., 1992) and Gpx activities (Aspberg and Tottmar, 1992; Buard et al., 1992; de Haan et al., 1992; Fan et al., 2003), a transient increase in manganese (Mn)-SOD activity in the mitochondria (Aspberg and Tottmar, 1992), and unchanged activity of glutathione reductase (Buard et al., 1992) contribute to an imbalance of brain antioxidative defense mechanisms early in maturation. High concentrations of unsaturated fatty acids, high rates of oxygen consumption, low concentrations of antioxidants, and availability of unbound redox-active iron contribute to vulnerability of the immature brain to oxidative damage. The limited ability of the developing brain to metabolize the high levels of $O_2^{\bullet-}$ and H_2O_2 under injurious conditions, such as H–I (Fullerton et al., 1998) or trauma (Tong et al., 2002; Fan et al., 2003), show that accumulation of H_2O_2 and lipid peroxides and lowered Gpx activity contribute to injury. Studies using transgenic animals provide further data that insufficiency in antioxidative enzymes in the developing brain impact injury tremendously since Gpx overexpression protects immature rat pups from H–I while

overexpression of CuZn-SOD makes injury worse (Sheldon et al., 2004). However, overexpression of CuZn-SOD is beneficial in adult animals after stroke (Chan et al., 1996). Under H–I conditions, an insufficient compensatory increase of Gpx activity and, hence, detoxification of H_2O_2 become a rate-limiting step (Fullerton et al., 1998), while conditions associated with increased Gpx activity result in protection of the neonatal brain (Sheldon et al., 2004; McLean et al., 2005). Not only neurons but also cells of other lineages, such as immature oligodendrocytes, are sensitive to H_2O_2 exposure, and Gpx activity affects outcome (Baud et al., 2004; Follett et al., 2004).

In neonatal rats, while pharmacological depletion of GSH, a substrate for Gpx, damages mitochondria and leads to mortality, systemic administration of a GSH precursor, glutathione monoethyl, reverses the injurious effect of GSH depletion (Martensson et al., 1991a, b). Low GSH levels lead to accumulation of intraparenchymal ROS, changes in cellular redox potential (Saitoh et al., 1998), and initiation of stress-activated signal transduction pathways (Kretz-Remy et al., 1996; Wilhelm et al., 1997; Li et al., 1998b). Studies in cultured neurons support in vivo data and show that diminished intracellular GSH concentrations affect the equilibrium between cytosolic and mitochondrial GSH (Makarov et al., 2002) and induce direct mitochondrial damage and neuronal death (Wullner et al., 1999; Vexler et al., 2003; Jiang et al., 2004).

Antioxidants tested in the ischemic developing brain include allopurinol, mannitol, methionine, and deferoxamine (Palmer, 1995; Sarco et al., 2000). A mixture of mannitol, methionine, and magnesium given after injury in the same model resulted in a shift to less severe injury, presumably by antioxidative effects (Mujsce et al., 1990). Allopurinol, a xanthine oxidase inhibitor, is protective after H–I in piglets (Peeters-Scholte et al., 2002, 2004) and rats (Palmer et al., 1993; Palmer, 1995). Deferoxamine, an iron chelator, affords protection after H–I in mice (Sarco et al., 2000) and rats (Palmer et al., 1994), presumably through a reduction in low-molecular weight iron and, hence, a decrease in the conversion of H_2O_2 to $^{\bullet}OH$ through the Fenton reaction as well as by stimulation of hypoxia-dependent genes (Mu et al., 2004). Deferoxamine is protective against excitotoxic (NMDA) and oxidative (H_2O_2) damage in primary cultures of hippocampal neurons (Almli et al., 2001).

3.6 Inflammation and Permeability of the Blood–Brain Barrier in Ischemic-Reperfusion Injury in the Neonatal Brain

The notion of immaturity has been applied to the blood–brain barrier (BBB), implying that at early developmental steps the BBB cannot effectively limit entrance of compounds as effectively as the mature BBB. The integrity of the mature BBB is controlled by a number of different and partially independent mechanisms, including the presence of extracellular matrix, tight junctions, and endothelial cells (Bolton et al., 1998; Romanic et al., 1998; Bazzoni et al., 2000). When intact, the BBB provides an almost complete block of the influx of immunoglobulins and complement. Importantly, formation of the BBB is largely complete early postnatally as tight junctions are present as soon as vessels are formed in the embryonic brain and endothelial cell transporters are in place (Schulze and Firth, 1992; Kniesel et al., 1996). Yet, the choroid plexus, where there is no BBB, permits passage of proteins into the CSF at birth, and regional transport declines as the brain matures (Saunders et al., 1999a, b).

Under pathophysiological conditions, such as H–I, focal ischemia, or inflammation, the integrity of the BBB and contributing mechanisms seem to differ greatly depending on age. In adult animals, ischemic injury to the brain parenchyma leads to a local production of proinflammatory cytokines (Barone et al., 1991; Feuerstein et al., 1998; Barone and Feuerstein, 1999) and chemotactic molecules (Baggiolini, 2001; Gerard and Rollins, 2001). Subsequently, there is an increased number of leukocytes in the systemic circulation (Anthony et al., 1998; Campbell et al., 2003), secondary changes in the adhesion properties of the surrounding vascular endothelium, and site-specific chemotaxis of circulating neutrophils (Barone et al., 1991; Garcia et al., 1994; Matsuo et al., 1995), followed by recruitment of macrophages and lymphocytes in the brain (Barone et al., 1991; Garcia et al., 1994). Activated neutrophils and monocytes accumulate in brain microvasculature, transmigrate into the ischemic tissue, and contribute to tissue damage likely by reducing local tissue perfusion through the release of free radicals, proteinases, and other cytotoxic substances (reviewed in Feuerstein et al., 1998; Jean et al., 1998; Rosenberg, 2002). Leukocytes (neutrophils, followed by monocytes) can plug the microvessels as early as 1–4 h after MCA

occlusion in adult rats (Garcia et al., 1994), and pretreatment with anti-CD18MAb IB41 can reduce the percentage of vessels exhibiting the "noreflow" phenomenon.

In immature rats, no neutrophil extravasation from blood vessels can be seen 24–96 h following H–I (Anderson et al., 1990; Palmer, 1995; Hudome et al., 1997; Bona et al., 1999; Palmer et al., 2004) or within 24 h after transient MCA occlusion in P7 rats (Wendland et al., 2003). However, permanent disruption of blood flow in combined permanent MCA occlusion and transient CCA occlusion is associated with neutrophil invasion into injured tissue (Benjelloun et al., 1999). Brain vascular permeability and leukocyte recruitment following interleukin-1β (IL-1β) or tumor necrosis factor α (TNFα) injections into the brain is age dependent and is much higher in 3-week-old animals compared to newborn or 3-month-old animals, and the effect is dependent on the presence of neutrophils (Anthony et al., 1997). Therefore, limited neutrophil infiltration may preserve tight junctions and limit protein entrance into injured neonatal brains. Eventhough unable to invade H–I tissue, neutrophils are capable of brain damage from within vessels (Hudome et al., 1997; Palmer et al., 2004) presumably by generation of oxygen free radicals, released lysosomal enzymes, myeloperoxidase, proteases, and other mediators.

Therefore, the emerging evidence suggests that inflammation plays an important role in neonatal H–I (McRae et al., 1995; Hagberg et al., 1996; Cowell et al., 2002, 2003) and focal ischemia–reperfusion (Benjelloun et al., 1999; Derugin et al., 2000). Cytokines exert a variety of biological functions including long-term developmental events, such as proliferation, differentiation, process extension, and cell survival (Patterson, 1993), as well as short-term events such as modulation of synaptic activity (Giovannelli et al., 1998) and participation in inflammatory responses. Leukocytes in the circulation, as well as tissue macrophages, are the main source for such proinflammatory cytokines as IL-1β, IL-6, and TNFα. Increased levels of these cytokines are thought to prime the endothelium by inducing adhesion molecules, including ICAM-1 and E- and P-selectins. Following H–I in P7 rats, the first signs of inflammation are seen within hours after an insult as is evident from the accumulation of multiple cytokines and chemokines (Szaflarski et al., 1995), including monocyte chemoattractant protein 1 (MCP-1) (Galasso et al., 2000a, b; Xu et al., 2001), macrophage inflammatory protein 1 (MIP-1) (Cowell et al., 2002), as well as activation of complement (Cowell et al., 2003; Ten et al., 2003, 2004). Injection of recombinant MCP-1 can exacerbate excitotoxic damage (Galasso et al., 2000a). Further proof-of-principle includes findings that deficiency in IL-1 converting enzyme attenuates H–I–induced MCP-1 expression and damage in the neonatal brain (Xu et al., 2001).

Microglia play a dual role in the brain: they protect the CNS against invading organisms and accumulation of debris after injury and amplify the effects of inflammation. Activation of microglial cells is a complex and multistep process that results in changes in morphological phenotype, release of inflammatory mediators, increased chemotactic and phagocytic activity, and antigen presentation (Carson et al., 1998; Raivich et al., 1999; Bohatschek et al., 2001). The timing and circumstance of activation depend on the settings of injury (infection, inflammation), the presence of various stimulants as well as the severity of injury. Following H–I or focal ischemia in neonatal rodents, activated microglia/macrophages rapidly accumulate in the injured tissue (Ivacko et al., 1996; Bona et al., 1999; Derugin et al., 2000; Cowell et al., 2002) and produce inflammatory cytokines (Cowell et al., 2002) and high levels of $NO^{\bullet}$ (Tsuji et al., 2000). Early and massive production of inflammatory molecules in injured pup brains may produce adverse effects. Drugs with broad antiinflammatory properties, such as minocycline (Arvin et al., 2002; Tsuji et al., 2004), pentoxifylline (Eun et al., 2000), and allopurinol (Palmer et al., 1993; Hudome et al., 1997), or inhibitors of the mitogen-activated protein kinase p38 (Hee Han et al., 2002), have shown protection in P7 rats following H–I. Timing of drug administration and species differences, however, may abolish (Tsuji et al., 2004) these neuroprotective effects, as with minocycline (Fox et al., 2005).

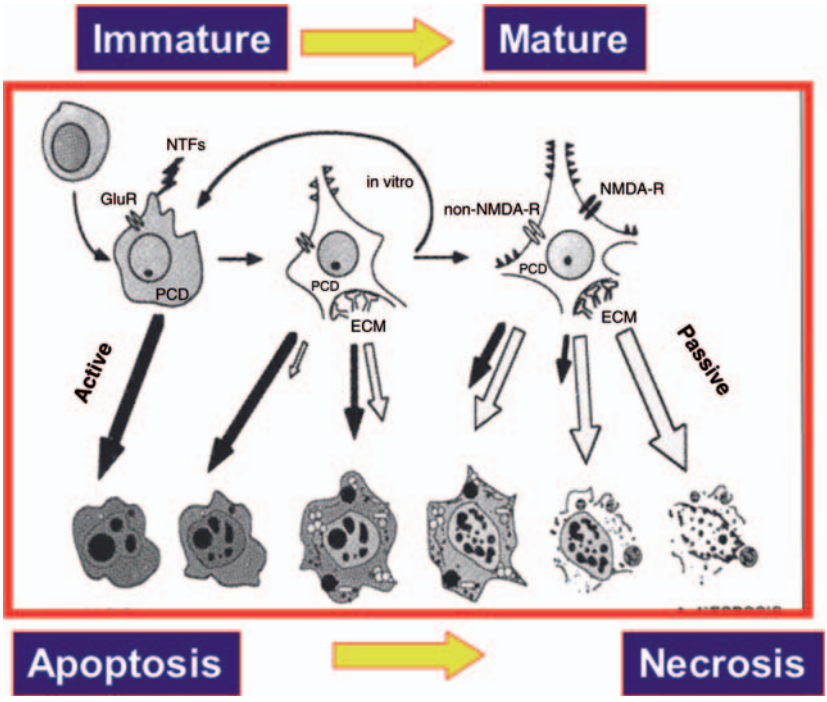
4 Molecular Mechanisms of Ischemic Cell Death in the Neonate

4.1 Mechanisms of Apoptotic and Nonapoptotic Cell Death in the Developing Brain

Programmed cell death is a part of normal brain development as neurons are overproduced during embryonic development and more than half of the neurons are lost in several regions (Raff et al., 1993;

Motoyama et al., 1995; Ikonomidou et al., 1999). Both necrosis and apoptosis after ischemia occur as modes of cell death, and the relative contribution of these modes depends upon injury severity (Lipton, 1999a; Dirnagl et al., 1999; Fiskum, 2004) and age (Cheng et al., 1997; Hu et al., 2000). In the immature brain, necrosis depends on injury severity following H–I, and a focal transient cerebral ischemia (Sheldon et al., 2001; Manabat et al., 2003) creates an intermediate form of death that exhibits features of both necrosis and apoptosis, a continuum or hybrid cell death (Martin et al., 1998; Puka-Sundvall et al., 2000; Northington et al., 2001a, b; Sheldon et al., 2001). A wide range of morphological phenotypes of dying neurons exist in the developing brain (*Figure 12-1*). While cell death is clearly associated with a diverse range of

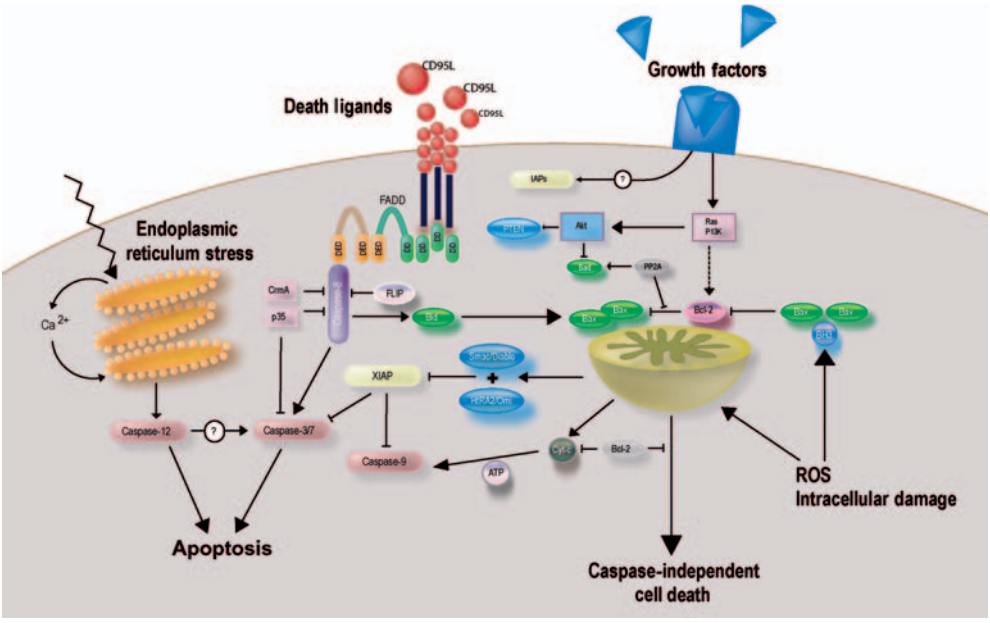
Figure 12-1
There is a wide range of morphological phenotypes of dying neurons in the developing brain. Adapted from Martin et al. (1998)



morphologies during early postnatal brain development, it is still debated whether apoptosis after injury is the same or different from programmed cell death in the normal developing brain.

There are several relatively independent apoptotic pathways that are either mediated via caspase-3 activation or mediated via caspase-independent pathways. (Raff et al., 1993; Motoyama et al., 1995; Ikonomidou et al., 1999). Caspase-3-dependent apoptotic pathways consist of an "intrinsic pathway" and an "extrinsic" pathway which are discussed in more detail elsewhere in this volume. Multiple apoptotic pathways are activated in the developing brain following an ischemic insult (*Figure 12-2*). The neonatal brain is thought to retain machinery for apoptotic cell death, which is readily activated following H–I (Han et al., 2000, 2002; Hu et al., 2000; Blomgren et al., 2001; Zhu et al., 2003, 2004), ischemia–reperfusion (Benjelloun et al., 1999; Manabat et al., 2003) and an excitotoxic stimulus (Ikonomidou et al., 1999). Compared with the adult brain, expression of many of the key components of apoptosis is high in the normal immature brain, including caspase-3 (Han et al., 2000, 2002; Hu et al., 2000; Blomgren et al., 2001;

Figure 12-2
Multiple caspase-dependent and -independent apoptotic pathways become activated in the developing brain following an ischemic insult. Details regarding the "intrinsic pathway" and the "extrinsic" pathway are discussed in the text. Illustrated by Sarah A. Drasner

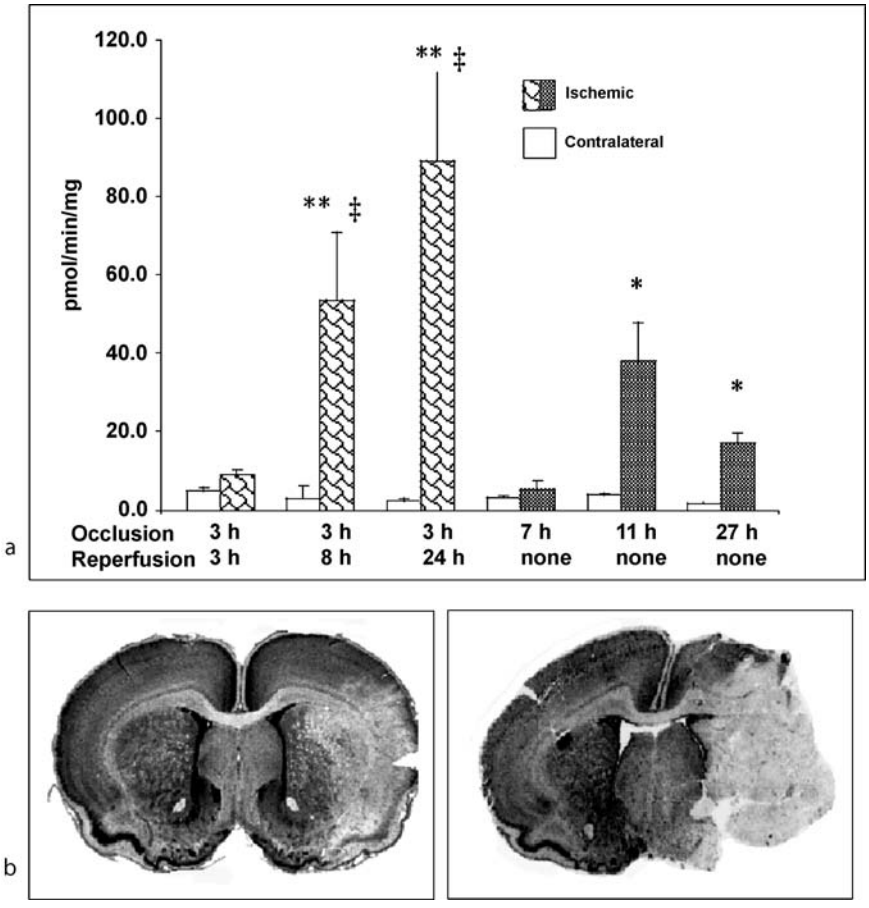


Zhu et al., 2003), Bcl-2 (Ferrer et al., 1997b; Yakovlev et al., 2001), Bax (Ferrer et al., 1997b; Northington et al., 2001a), apoptosis protease activating factor-1 (Apaf-1) (Yakovlev et al., 2001; Ota et al., 2002), and apoptosis-inducing factor (AIF) (Zhu et al., 2003). At the same time, activation of the executional caspase, caspase-3, in the brain is maximal during the first postnatal week (Hu et al., 2000; Wang et al., 2001; Yakovlev et al., 2001) and declines rapidly after P7–P14. Expression of cytochrome *c*, cytochrome oxidase (Zhu et al., 2003), and possibly caspase-9 (Zhu et al., 2003) change reciprocally with age. Cytochrome *c*, Apaf-1, caspase-9, and caspase-3 have been shown to form an evolutionarily conserved cell death pathway in the mammalian nervous system (Kuan et al., 2000): caspase-9 binds to Apaf-1 and cytochrome *c* is released from mitochondria forming an active apoptosome (Green and Reed, 1998) that activates caspase-3. Activated caspase-3, in turn, is capable of cleaving a number of targets, including inhibitor of caspase-activated DNase (ICAD) and poly-ADP-ribose polymerase (PARP) (Wang et al., 2001; Hagberg et al., 2004).

Under ischemic conditions, caspase-3 apoptotic death machinery can be turned on and the contribution of caspase-dependent neuronal death is much more profound in immature than in adult ischemic brains (Hu et al., 2000). A robust increase in caspase-3 activity occurs in the injured tissue following H–I (Han et al., 2000, 2002; Hatai et al., 2000; Hu et al., 2000; Lankiewicz et al., 2000; Nakajima et al., 2000; Blomgren et al., 2001) or focal transient MCA occlusion at P7 (Benjelloun et al., 2003; Manabat et al., 2003; Joly et al., 2004). Caspase-3 activation occurs both in the ischemic core and in regions perifocal to injury following focal ischemia (Manabat et al., 2003) and depends on the presence of reperfusion (Manabat et al., 2003). Recirculation of ischemic regions of the neonatal brain appear important for caspase-dependent neuronal death since 15- to 18-fold increase in caspase-3 activity is seen 24 h following transient MCA occlusion, while a diminished and earlier peak in caspase-3 activity (eightfold increase) and a higher incidence of neurons dying by necrosis is reported after permanent MCA occlusion. (Manabat et al., 2003) (*Figure 12-3*). Diminished mitochondrial function and energy failure after ischemia are likely to account for these differences since apoptosis is an energy-requiring process. Both a peptide-based pan-caspase inhibitor (Cheng et al., 1998) and a selective and potent reversible caspase-3 inhibitor M826 (Han et al.,

Figure 12-3

Caspase-3 activation occurs both in the ischemic core and in the regions perifocal to injury following focal ischemia and depends on the presence of reperfusion. (a) Caspase-3 activity was measured in an Asp-Glu-Val-Asp-aminomethylcoumarin (DEVD-AMC) cleavage assay using Ac-DEVD as a substrate. Enzyme activity is expressed as pmol/min/mg protein. *$p < 0.01$ values in injured tissue versus values in the matching regions of the contralateral hemisphere, **$p < 0.004$ values in injured tissue versus values in the matching regions of the contralateral hemisphere, ‡$p < 0.0001$ values in injured tissue following permanent MCA occlusion versus values in the same hemisphere following the same duration of transient MCA occlusion. (b) Severity of injury 24 h after transient (*left*) and 27 h after permanent (*right*) MCA occlusion was determined by cresyl violet staining. It should be noted that more severe injury following permanent MCA occlusion is accompanied by less caspase-3 activity (a). Adapted from Manabat et al. (2003)



2002) have provided protection against neonatal H–I brain injury while leaving calpain-dependent excitotoxic/necrotic cell injury unaffected.

Another component of caspase-mediated apoptotic cell death depends on the FAS death receptor-mediated activation of caspase-8 and is relatively independent of mitochondrial function (Northington et al., 2001a, b). Caspase-8, not caspase-9-induced activation of caspase-3, is found to be profound in early ischemic cell death in adults (Benchoua et al., 2001; Morita-Fujimura et al., 2001), implicating the death receptor in this process. In neonatal animals, FAS-dependent neuronal death has been shown to occur in a delayed fashion in the thalamus, an area remotely located from the ischemic core, and lack of functional Fas-death receptors protects

the brain from H–I (Graham et al., 2004). Before the appearance of large numbers of apoptotic profiles in the ventral-basal thalamus, expression of Fas-death receptor protein, activated forms of caspase-8 and caspase-3, and proapoptotic Bcl-2 proteins are increased (Northington et al., 2001a).

AIF is another factor that when translocated from mitochondria to the nucleus, induces chromatin condensation and DNA fragmentation (Daugas et al., 2000). Its expression in the brain seems to be independent of postnatal age (Zhu et al., 2003). Following H–I, AIF translocation from mitochondria reportedly occurs before the release of cytochrome *c* and the resultant DNA damage and neuronal death is unaffected by caspase-3 inhibitors, suggesting that AIF mediates apoptosis, which is independent of caspase activation (Zhu et al., 2003) and contributes to rapid cell death following H–I (Matsumori et al., 2003). PARP-1 inhibitors or genetic deletion of PARP-1 abort AIF-mediated neuronal death in NMDA-treated cortical neurons, suggesting that AIF-mediated responses lie downstream of PARP-1 activation (Yu et al., 2002).

4.2 The Role of Mitochondria in Mediating Ischemic Cell Death in the Neonatal Brain

Mitochondria are the primary intracellular targets of the postischemic reduction of available oxygen and glucose, as well as direct targets of NMDA- and ROS-induced elevation of $[Ca^{2+}]_i$, with resultant changes in structure and function. Mitochondria play a central role in initiating cell death programs via release of several major proapoptotic proteins in the cytosol as well as by affecting necrotic cell death. During normal brain development, activities of mitochondrial enzymes and mitochondrial content undergo changes (Holtzman and Moore, 1975; Dienel et al., 1977; Sitkiewicz et al., 1982; Keelan et al., 1999). The respiration rate is overall lower in brains of animals younger than 1 month and reaches a maximum in adult animals (Holtzman and Moore, 1975), possibly predisposing mitochondria to ischemic insult early postnatally. Studies using neonatal H–I models have shown changes in mitochondrial respiration, energy metabolism, and redox potential (Yager et al., 1991, 1996; Gilland et al., 1998b; Keelan et al., 1999; Puka-Sundvall et al., 2000). The capacity of the brain mitochondria to take up Ca^{2+} also depends on age: in the presence of ATP, mitochondria from P16–P18 brain have lower capacity, while the immature brain mitochondria exhibit greater Ca^{2+} uptake capacity in the absence of ATP, indicating a greater resistance of the immature brain mitochondria to Ca^{2+}-induced dysfunction under conditions relevant to acute ischemic brain injury (Robertson et al., 2004).

Three of the known apoptotic pathways are directly dependent on mitochondria: the intrinsic and extrinsic pathways of caspase activation and a pathway involving AIF. The intrinsic pathway triggers the release of cytochrome *c*, Apaf-1, and procaspase-9 in the cytosol, producing active caspase-9 (Green and Reed, 1998; Kroemer et al., 1998; Li et al., 1998a) as well as a second mitochondrial-derived activator of caspase/direct inhibitor of apoptosis (IAP)-associated binding protein with low PI (Smac/DIABLO) and HtrA2/Omi, which antagonizes IAP proteins. The extrinsic pathway of caspase activation is triggered by binding of death ligand Fas to its death receptor followed by cleavage of caspase-8 to its active form, which then directly activates caspase-3 and truncates a proapoptotic member of the Bcl-2 family protein, Bid, causing translocation to mitochondria and death (Li et al., 1998a).

The third pathway involving AIF is independent of the other pathways, as well as cytochrome *c* release, and contributes directly to DNA fragmentation. The role of several members of the Bcl-2 family proteins, including Bid, Bad, Bcl-xL, and Bak, in the regulation of inner mitochondrial permeability transition, cellular redox state, and oxidative damage-induced cell death as a part of normal development and during injury is summarized in several recent reviews (Reed, 1997; Green and Reed, 1998; Fiskum, 2004). High expression of mitochondrial Bax (Polster et al., 2003), a protein that mediates cytochrome *c* release and apoptosis during neurodevelopment, may explain the increased susceptibility of immature neurons to pathological processes like H–I (Ferrer et al., 1994; Ferrer et al., 1997b; Gibson et al., 2001; Northington et al., 2001a). The balance between different members of the Bcl-2 family proteins, which is changing during development, can profoundly affect death depending on the specific death stimulus, neuron subtype, and stage of postnatal development (Reed, 1997; Green and Reed, 1998; Fiskum, 2004). Some members, like

Bak, can switch during development from being anti- to proapoptotic (Fannjiang et al., 2003). DNA repair in mitochondria also declines with age (Chen et al., 2002).

Importantly, mitochondria from astrocytes and neurons have very different properties. Compared with neurons, astrocytes are less dependent on oxidative phosphorylation. Following ischemia, both synaptic and nonsynaptic mitochondria exhibit a loss of respiratory activity, but the degree of inhibition of respiratory chain complex I necessary for inhibition of oxidative phosphorylation is 25% for CA1 synaptic mitochondria compared with 60% for nonsynaptic mitochondria (Davey et al., 1997). Changes in the mitochondrial permeability transition pore in neurons and astrocytes are different as well (Bambrick et al., 2004) and preferential neuronal susceptibility to oxygen–glucose deprivation may, in part, depend on these differences (Papadopoulos et al., 1997).

4.3 Gene and Protein Expression as Modulators of Ischemic and Hypoxic–Ischemic Injury in the Immature Brain

Many factors that control programmed neuronal cell death or neuronal survival are developmentally regulated, including growth factors (Knusel et al., 1994; Cheng et al., 1997), receptor systems (Ikonomidou et al., 1989; McDonald and Johnston, 1990; McDonald et al., 1992; Ikonomidou et al., 1999), and transcription factors that play an important role in the signal transduction cascades between extracellular messengers and long-term changes in cellular responses (Ferrer et al., 1997a; Walton et al., 1999). Following an H–I insult in the neonatal brain, induction of genes coding for growth factors and their associated binding proteins and receptors (Beilharz et al., 1993; Klempt et al., 1993b; Gustafson et al., 1999) as well as rapid changes in the levels of early immediate genes, such as members of the AP-1 dimeric complex c-Jun, c-Fos, and ATF-2 (Walton et al., 1999), have been documented. Exogenous stimulation of prosurvival mechanisms using growth factors NGF, BDNF, and bFGF, but not NT-3 (Gluckman et al., 1992; Kirschner et al., 1995; Kirschner et al., 1996; Cheng et al., 1997; Han and Holtzman, 2000), with the consequent change in transcriptional responses are reported to have a neuroprotective effect in experimental models of H–I. Induction of several heat-shock proteins, including HSP72 (Ferriero et al., 1990) and HSP32 (hemeoxygenase) (Bergeron et al., 1997), have been shown post H–I. Increase in heat-shock proteins is considered as a stress response and can serve both as indicators of injury severity (Sharp et al., 2000) and as mediators of ischemic injury, playing a dual role in injury (Bergeron et al., 1997).

Nonsynchronized changes in expression of proteins in response to injury, as well as a complex nonlinear relationship between spatial and temporal changes in cells, affect the response. The same protein can play opposite roles in triggering or propagating injury. Downstream effects from a single protein are frequently regulated by multiple parallel pathways in the same cell. Therefore, in addition to studying the role of a particular gene/protein candidate on ischemic outcome, ischemic tolerance has been used as one of the ways to understand neuroprotection. Several different approaches have been used. Hyperthermia (Sato et al., 2000), spreading depression and exposure to metabolic inhibitors or inflammatory stimuli, brief ischemia (Stenzel-Poore et al., 2003), or hypoxia (Gidday et al., 1994; Jones and Bergeron, 2001) provide protection, including after H–I in neonatal rodents (Gidday et al., 1994; Jones and Bergeron, 2001). The precise molecular mechanisms involved in the development of tolerance against ischemic damage are unknown, and so far several studies in adult (Shamloo et al., 1999; Yano et al., 2001) and neonatal (Jones and Bergeron, 2001) animals have pointed at altered gene expression, particularly, hypoxia-inducible/regulated genes (Semenza, 2000b; Bernaudin et al., 2002; Sharp and Bernaudin, 2004), and activation of various intracellular signaling pathways, including mitogen-activated protein kinases (MAPKs) and phosphatidylinositol-3-kinase (PI3K).

One gene implicated in the process of preconditioning is hypoxia-inducible factor 1 (HIF1). This transcription factor consists of two subunits, HIF1α and HIF1β. While HIF1β is expressed constitutively in all cells and does not respond to changes in oxygen tension, HIF1α limits initiation of hypoxia-induced transcriptional changes, since under normoxic conditions HIF1α is rapidly degraded via prolylhydroxylase-dependent proteosomal mechanisms. Hypoxia stabilizes HIF1α, and upon its accumulation and phosphorylation, HIF1α dimerizes with HIF1β, makes heterodimer with p300/CBP, which enables activation

of hypoxia response elements (HRE) in HIF target genes. HIF1 targets include genes that produce proteins that participate in energy metabolism, angiogenesis, erythropoiesis, iron metabolism, and cell death (reviewed in Semenza, 2000a; Sharp and Bernaudin, 2004).

Induction of HIF1α has been reported in the ischemic penumbra (Bergeron et al., 1999) in adult rats and in the ischemic core and penumbra following neonatal focal ischemia (Mu et al., 2003). Increased expression of HIF1-dependent genes, such as the vascular endothelial growth factor (VEGF) gene (Hayashi et al., 1997; Mu et al., 2003), genes of several glycolytic enzymes (Sheldon et al., 1998; Bergeron et al., 1999), GLUT1 (Bergeron et al., 1999), and the erythropoietin (EPO) gene (Matsushita et al., 1998; Aydin et al., 2003; Mu et al., 2003; Chang et al., 2004; Kumral et al., 2004) are thought to be important in recovery from H–I and ischemia-reperfusion in the neonatal brain.

4.4 Mitogen-Activated Protein Kinases

The family of MAPKs consists of subfamilies of extracellular-activated protein kinases (ERK1/2) and stress-activated protein kinases p38 and c-Jun N-terminal kinase (JNK). Relative activation of the ERKs, p38, and JNKs regulates a variety of cell functions, including cell survival or death (Xia et al., 1995; Kummer et al., 1997). Specificity of responses is accomplished by the presence of multiple parallel modules for transducing signals and a complex hierarchy of regulation that occurs on both transcriptional and translation levels. Different durations of specific MAPKs activation have been suggested as a general theme to control cell fate (Xia et al., 1995). While neuronal ERK1/2 is implicated in protection against neuronal cell death, contrasting effects of ERK1/2 activation in vitro and in vivo have been observed (reviewed in Irving and Bamford, 2002). Early/transient and late/sustained activations of JNK/p38 induced by TNFα (Guo et al., 1998; Roulston et al., 1998), ultraviolet radiation, growth factor withdrawal (Xia et al., 1995), and cerebral ischemia (Herdegen et al., 1998; Mielke et al., 1999; Irving et al., 2000; Legos et al., 2001) have been implicated in cell survival and apoptosis. The pathophysiological role of the MAPK p38 is generally viewed as injurious after cerebral ischemia as pharmacological inhibition of this kinase reduces postischemic cell death (Sugino et al., 2000), infarct size, and neurological deficits (Legos et al., 2001), and its activation contributes to microglia-mediated ischemic injury (Walton et al., 1998).

ERK1/2 phosphorylation occurs rapidly in neurons within damaged areas and, later, in astrocytes, microglial cells, and progenitor cells after H–I in P7 rats (Wang et al., 2003). ERK1/2 activation has been shown to mediate protection by BDNF after H–I (Han et al., 2000) since pharmacological inhibition of ERK, not PI3K pathway, inhibits the ability of BDNF to block H–I-induced caspase-3 activation and tissue loss (Han and Holtzman, 2000). Because ERK1/2 can be activated, often in the same cell type, by both prosurvival factors, such as BDNF (Hetman et al., 1999) and toxic stimuli (Xue et al., 2000), ERK1/2 activation alone is not predictive of cellular responses. While JNK phosphorylation decreases in the hippocampus and cortex immediately following H–I, a transient increase of MAPK p38 phosphorylation occurs in both injured regions immediately following H–I (Hee Han et al., 2002), and inhibition of p38 before H–I significantly protects the neonatal brain from H–I injury. It is not known if protection is associated with attenuation of cytokine production, the known pathophysiological mechanism of the MAPK p38 (Saccani et al., 2002), but p38-mediated injury seems to be independent of caspase-3 activation (Hee Han et al., 2002). Hypoxic preconditioning studies, where a brief hypoxic period 24 h before H–I protected P7 rats against injury, showed that MAPKs play different roles in ischemic tolerance as activation of ERK1/2, but not JNK or p38, signaling induced hypoxia tolerance in the neonatal rat brain (Jones and Bergeron, 2004).

4.5 Susceptibility of Nonneuronal Cells to Hypoxic and Ischemic Injury During Development

The majority of studies of cell death following cerebral ischemia have focused on neurons. However, both in vitro and in vivo findings point to the susceptibility of astrocytes (reviewed in Aschner et al., 2002; Bambrick et al., 2004) and oligodendrocytes (Back et al., 1998, 2002; McQuillen and Ferriero, 2004) to

ischemia and hypoxia. Neuroglial interactions play an important role in protecting neurons by providing GSH (Dringen et al., 2000), preventing glutamate toxicity through glutamate uptake (Anderson and Swanson, 2000), and by contributing to the integrity of the BBB via endothelial-astrocyte interactions (Ballabh et al., 2004). Diminished brain GSH levels (Sheldon et al., 2004) and elevated extracellular glutamate post H–I are detrimental to the immature brain (Gurd et al., 2002). While neurons are by far more susceptible to oxygen–glucose deprivation (OGD) than astrocytes, a nonlethal duration of OGD causes decrease in astrocyte mitochondrial membrane potential and loss of cytochrome *c*, whereas lethal duration of OGD causes loss of GSH, oxidative damage, and impaired glutamate uptake (Papadopoulos et al., 1997).

Loss of astrocytes and the morphological changes in remaining astrocytes are very rapid within injured brain regions, but these changes are often overlooked (Zhao et al., 2003). GFAP-immunoreactive astrocytes are seen adjacent to injured tissue by 24 h after transient focal ischemia in P7 rats, but very few GFAP-positive cells are present in the ischemic core (Derugin et al., 2000). Combined permanent MCA occlusion and transient CCA occlusion in P7 rats result in caspase-3-dependent death of GFAP-immunoreactive astrocytes starting at 48 h (Benjelloun et al., 1999, 2003). Many astrocytes expressed Bax (Benjelloun et al., 1999), released cytochrome *c* from mitochondria, and showed caspase-3 activation (Benjelloun et al., 1999, 2003) and, later, DNA fragmentation and PARP cleavage (Ducrocq et al., 2000; Joly et al., 2003).

The contribution of white matter injury to H–I brain damage is maturation dependent and depends on the appearance of oligodendrocyte progenitors (Back et al., 1998, 2002). While late oligodendrocyte progenitors are the major oligodendrocyte lineage that die by apoptosis, early oligodendrocyte progenitors and more mature oligodendrocytes are highly resistant (Back et al., 1998, 2002; Levison et al., 2001). Immature oligodendrocytes are highly vulnerable to H–I injury, but newly produced oligodendrocytes are in abundance in the injured striatum, corpus callosum, and the infarct core 3–4 weeks following H–I at P7 (Zaidi et al., 2004).

Oxidative stress and excitotoxicity are two mechanisms that predispose oligodendrocyte progenitors to ischemic death, as has been demonstrated in vitro by GSH depletion and exposure to exogenous free radicals (Back et al., 1998; Follett et al., 2004). Developmental differences in enzymes that regulate GSH levels, AMPA receptors, as well as differences in levels of antioxidants largely account for stage-dependent susceptibility of cells of oligodendrocyte lineage to H–I.

Another cell type susceptible to H–I insult is subplate neurons (reviewed in McQuillen and Ferriero, 2004). Subplate neurons are a transient population in the developing neocortex and are involved in the formation of area-specific thalamocortical connections (McQuillen et al., 2002). Subplate neurons undergo programmed cell death in the first postnatal week in mice (McQuillen et al., 2002). H–I-induced death of this cell population is believed to contribute to white matter damage in the very immature brain. Mechanisms of selective vulnerability of subplate neurons are not very well understood, but expression of NMDA-R1, AMPA, and kainate receptors (Catalano et al., 1997; Furuta and Martin, 1999) and the presence of the readily available cell death machinery may make these cells prone to excitotoxic and oxidative injury.

5 Neurogenesis as a Repair Mechanism in Postischemic Neonatal Brain

Although plasticity is a common term used in relation to the immature brain, little is known about the scope of endogenous neurogenesis and gliogenesis after neonatal ischemia and its role in repair and restoration of function. Cell proliferation is high during normal embryogenesis and gradually declines during the postnatal period. Subventricular zone (SVZ)-olfactory bulb neurogenesis peaks during the first 2 postnatal weeks (Bayer, 1983) but persists throughout life in the rodent (Lowenstein and Parent, 1999). In the adult, neurogenesis increases after insults like cerebral ischemia or excitotoxic stimuli (Parent et al., 2002; Ekdahl et al., 2003; Monje et al., 2003), and newly produced neurons migrate to injured regions and differentiate into region-appropriate phenotypes, suggesting that the mature brain is capable of replacing some neurons lost after ischemic injury (Parent et al., 2002). The ability of the adult injured brain to replace

neurons is adversely affected by inflammation (Ekdahl et al., 2003; Monje et al., 2003), raising the possibility that endogenous repair mechanisms are limited.

In the neonatal brain, neurogenesis also occurs following both ischemic (Chang et al., 2004) and H–I (Plane et al., 2004) insults. An increase in cell proliferation in the SVZ ipsilateral to the insult is seen as early as 2 weeks after injury (Plane et al., 2004); it increases inversely with elevation of injury severity within the moderate range of injury (Chang et al., 2004; Plane et al., 2004) and diminishes when injury is severe (Levison et al., 2001). Newly generated cells in the injured neostriatum express markers of medium spiny neurons, which characterize most of the neostriatal neurons lost after the insult (Plane et al., 2004). H–I also stimulates cell proliferation and neurogenesis in the SVZ and peri-infarct striatum. Some newborn cells express a neuronal phenotype at P24, but not at P31, indicating that neurogenesis may be short lived (Plane et al., 2004). EPO, which is known to promote neurogenesis in vitro (Shingo et al., 2001) and enhance neurogenesis in SVZ following stroke in adult rodents (Wang et al., 2004), markedly preserves hemispheric volume and significantly improves the asymmetry of forelimb use 2 weeks following transient MCA occlusion in P10 rats, and decreases the expansion of SVZ ipsilateral to the occlusion compared to placebo-treated pups (Chang et al., 2004). The ability of EPO to limit SVZ expansion and promote neurogenesis while improving histological and functional outcomes following neonatal stroke suggests a role for repair.

6 Summary

Understanding the molecular mechanisms underlying the injury response after ischemia in the developing nervous system will allow the rational development of effective therapies. It is unclear whether to target protective mechanisms (via preconditioning), salvaging mechanisms (via "neuroprotective pharmacological therapies), or repair mechanisms (via neurogenesis). Clearly, combination therapies will be required and possibly therapies given at different times (perhaps late) during injury evolution are needed. Recent data on the combined use of hypothermia and topiramate (a drug that decreases AMPA-regulated excitotoxicity) reveal exciting enhanced protection over a single modality (Liu et al., 2004). Encouraging results from a trial of hypothermia for H–I in the term newborn will pave the way for future trials for stroke and H–I in the young brain in the twenty-first century (Gluckman et al., 2004).

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13 Molecular Mechanisms for Early Brain Injury After Subarachnoid Hemorrhage

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Abstract: An increasing volume of experimental and clinical data indicates that early brain injury after initial bleeding largely contributes to unfavorable outcome and mortality after subarachnoid hemorrhage (SAH). Patients who recover from cerebral ischemia caused by intracranial pressure (ICP) raised over diastolic blood pressure suffer from severe injury to the brain tissues and cerebral microvasculature. This chapter aims to review: (1) pathophysiological factors and molecular agents acting on cerebral and vascular tissues after initial bleeding; (2) molecular responses to the early intracranial phenomena in SAH; (3) molecular signaling that contributes to the early development of pathological sequelae of SAH, including blood–brain barrier rupture, brain edema, and apoptosis; and (4) molecular mechanisms of cell death in the brain occurring early after SAH.

List of Abbreviations: 20-HETE, 20-hydroxyeiscosatetraenoic acid; $5HT_{1B}$, 5-hydroxytryptamine 1B; AMPA, alpha-amino-3-hydroxy-5-methylisoxasole-4-propionate; AQP1, aquaporin-1; ATP, adenosine triphosphate; BBB, blood–brain barrier; BNIP3, E1B 19K/Bcl-2-binding protein Nip3; CBF, cerebral blood flow; cGMP, cyclic guanosine monophosphate; CGRP, calcitonin gene-related peptide; COX-2, cyclooxygenase-2; CPP, cerebral perfusion pressure; CSF, cerebrospinal fluid; DAG, diacylglycerol; ERK1/2, extracellular signal-regulated kinase 1/2; ET-1, endothelin-1; FADD, Fas-associated protein with death domain; GPCR, G protein–coupled receptors; GSH-Px, glutathione peroxidase; HIF-1, hypoxia-inducible factor 1; ICP, intracranial pressure; IL-1β, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; LPA, lysophosphatidic acid; LTC4, leukotriene C4; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase; MMP-9, matrix metalloproteinase-9; NFκB, Nuclear Factor kappa B; NIX, Nip3-like protein X; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; PARP, poly(ADP-ribose) polymerase; PDEV, phosphodiesterase enzyme type V; PDGF, platelet-derived growth factor; PG6-keto F1α, 6-keto-prostaglandin F1α; PGE2, prostaglandin E2; PGF2α, prostaglandin F2α; PI3K, phosphatidylinositol-3 kinase; PKC, protein kinase C; PLC, phospholipase C; PP1, Src-family tyrosine kinase inhibitor; PTK, nonreceptor protein tyrosine kinase; RIP1, receptor-interacting protein-1; SAH, subarachnoid hemorrhage; SH2, Src-homology 2; SOD, superoxide dismutase; SP, substance P; TNF-α, tumor necrosis factor α; TRAF, TNF receptor-associated factor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; TXA2, thromboxane A2; TXB2, thromboxane B2; VEGF, vascular endothelial growth factor

1 Introduction

Spontaneous subarachnoid hemorrhage (SAH) represents 5%–7% of all strokes,and about 30,000 Americans will suffer SAH each year (Kassell et al., 1985; Broderick et al., 1992). The overall death rate in 30 days is 50%, and significant complications occur in 50% of individuals who survive SAH (Winn et al., 1983; Broderick, 1993; Kaptain et al., 2000; Kissela et al., 2002). No effective treatment is available for early brain injury after the initial bleeding (O'Hare, 1987). Factors responsible for the impact of the initial bleeding in SAH include raised intracranial pressure (ICP), decrease in cerebral blood flow (CBF) and cerebral perfusion pressure (CPP), blood–brain barrier (BBB) rupture, brain swelling, brain edema, acute vasospasm, and impaired autoregulation. The molecular mechanisms and basic signaling pathways for these factors are also responsible, particularly when cellular and extracellular compartments are involved.

2 Cerebral Ischemia in Early Brain Injury

Elevation of ICP and acute vasoconstriction, especially at the microcirculation level, are the two major factors that reduce CBF and CPP after initial bleeding in SAH and lead to global ischemia (Bederson et al., 1998). Multiple pathogenic factors have been described for the reduction of CBF after the initial bleeding. First, hemoglobin released into the subarachnoid space is involved in the pathogenesis of cortical spreading ischemia, characterized by cortical blood flow below ischemic threshold, propagating together with neuronal and astroglial depolarization waves (Petzold et al., 2003). Hemoglobin has an inhibitory effect

on the Na^+/K^+-ATPase activity, which plays an important role in the mechanism of depolarization (Sadrzadeh et al., 1987). Second, endothelin-1 (ET-1) released from cerebral arteries reduces Na^+/K^+-ATPase activity and works in concert with hemoglobin in the induction of cortical spreading ischemia (Petzold et al., 2003). Third, activation of 5-hydroxytryptamine 1B ($5\text{-}HT_{1B}$) receptors induces formation of hydroxyeicosatetraenoic acid (HETE) that produces potent vasoconstriction and reduction in CBF (Cambj-Sapunar et al., 2003). Fourth, immediate reduction of nitric oxide availability after the initial bleeding is linked to the "sink-effect" of hemoglobin, that scavenges nitric oxide (Sehba et al., 2000; Schwartz et al., 2000b). Fifth, elevations of ICP and global hypoxia/ischemia immediately after the initial bleeding impair autoregulation, decrease CBF, and may contribute to brain swelling (Niikawa et al., 1998). Sixth, the impact of the initial bleeding enhances the production of superoxide anion catalyzed by NADPH oxidase (Kim et al., 2002). Both endothelial and smooth muscle cells express NADPH oxidase that generates superoxide radicals (Lassegue and Clempus, 2003). CuZn-superoxide dismutase (CuZn-SOD) gene transfer after the initial bleeding restored blunted vasodilation of the pial artery in response to calcitonin gene-related peptide (CGRP) and K^+ channel opener levcromakalim (Shin et al., 2003). Finally, early brain injury activated tyrosine kinase, that inhibits K^+ channels resulting in vasoconstriction (Sobey and Faraci, 1998; Hong et al., 2001), although it remains to be determined whether K^+ has a role in early brain injury after the initial bleeding.

One of the molecular factors mediating the ischemic signals is hypoxia-inducible factor-1α (HIF-1α). HIF-1α is a transcription factor specifically activated by hypoxia (Jin et al., 2000; Chavez and LaManna, 2002). The HIF family is composed of two subunits: the hypoxia-regulated α subunit HIF-1α (or its paralogs HIF-2α and -3α) and the oxygen-insensitive β subunit HIF-1β [arylhydrocarbon receptor nuclear translocator (ARNT)] (Semenza et al., 2000). Although accumulation of HIF-1α in the ischemic or hypoxic tissues might promote adaptive mechanisms for cell survival (Bergeron et al., 1999), high levels of HIF-1α might bind with tumor suppressor p53 to activate apoptotic pathways in the brain tissues after cerebral ischemia (Carmeliet et al., 1998; Minet et al., 2000; Volm and Koomagi, 2000; Brune et al., 2001; Moritz et al., 2002; Goda et al., 2003) and traumatic brain injuries (Halterman and Federoff, 1999; Yu et al., 2001). It is very likely that the level of HIF-1α is elevated in the brain tissues during early brain injury after the initial bleeding (*Figure 13-1*) as HIF-1α is increased after global or focal cerebral ischemia (Bergeron et al., 1999; Chavez and LaManna, 2002), and HIF-1α may participate in the pathogenesis of early brain injury.

Among HIF-1 target genes, Bcl-2/adenovirus EIB 19-kDa-interacting protein 3 (BNIP3) and Nip3-like protein X (NIX) mediate cell death on hypoxia. BNIP3 is a proapoptotic member of the Bcl-2 family. Increased BNIP3 expression was reported to occur in hypoxia and global cerebral ischemia exceeding a 10-min duration but does not in the chronic moderate ischemia (Schmidt-Kastner et al., 2004). Recently BNIP3 positive-stained cells and increased protein content were found in vulnerable sector CA1 of the hippocampus in the perforation model of SAH accompanied by significant HIF-1α upregulation and morphological features of profound cell injury (Ostrowski et al., 2005).

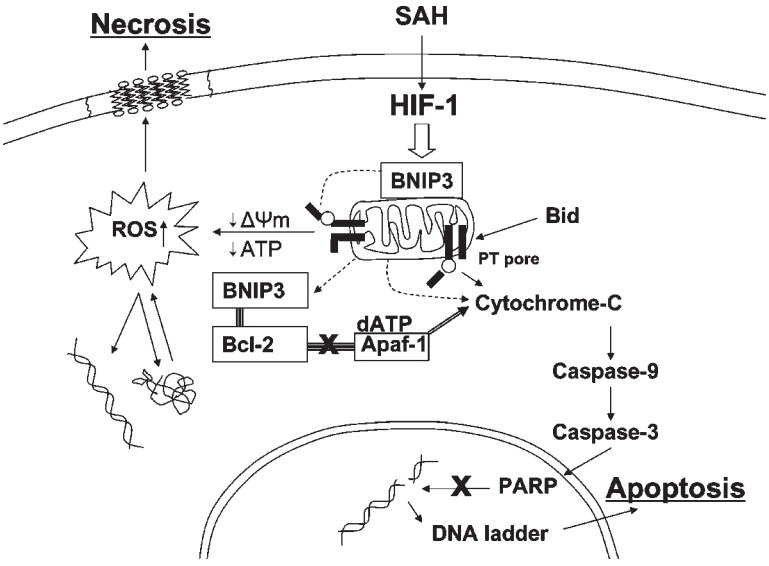
Other aspects of cerebral ischemia, including membrane depolarization in the penumbra, Ca^{2+} accumulation in the nerve cells, and glutamate receptor activation, they are present after the initial bleeding. Indeed, glutamate levels were elevated 600% in the hippocampus and cortex (Bederson et al., 1998), and Ca^{2+} accumulation and membrane depolarization were documented in cerebral arteries after SAH (Zubkov et al., 2003). Elevation of Ca^{2+} promotes the formation of nitric oxide (Suzuki et al., 1995) and other reactive oxygen species (Imperatore et al., 2000), dysfunction of mitochondria (Marzatico et al., 1990, 1993), activation of proteases (Zhang et al., 2003), and inflammation (Dumont et al., 2003). All of these processes contribute to the disintegration of vital cellular elements, such as the cytoskeleton, cell membranes, and nuclear DNA, after SAH. Some of these events have been studied, but while most of them are still poorly understood.

3 Brain Edema in Early Brain Injury

One of the early indications of brain injury after SAH is the alteration of BBB permeability (Peterson and Cardoso, 1983; Doczi et al., 1986; Sasaki et al., 1986; Zuccarello and Anderson, 1989; Nakagomi et al., 1989b;

Figure 13-1

The role of BNIP3 in apoptosis and mechanisms of necrosis-like cell death after SAH are shown. Ischemia associated with SAH activates HIF-1α and its target genes, including BNIP3, which opens the mitochondrial permeability transition pore; however, it remains controversial whether BNIP3 induces release of cytochrome *c*, which combines with Apaf-1 and activates caspase-9. Caspase-3 translocates into the nucleus and cleaves PARP, thus impairing DNA repair. The mechanism of cell death includes BNIP3 heterodimerization with antiapoptotic molecules, including Bcl-2 and their sequestration. On BNIP3-mediated cell death, membranes are disrupted, displaying early increase of permeability. Suppression of the proton electrochemical gradient and increased free radical production may underlie the mechanism of necrosis-like damage



Johshita et al., 1990a; Germano et al., 2000; Imperatore et al., 2000). Dysfunction of BBB contributes to brain edema (Johshita et al., 1990b; Doczi et al., 1995; Laszlo et al., 1995) and prolongs elevated ICP values after SAH (Hayashi et al., 1977; Voldby and Enevoldsen, 1982; Fornezza et al., 1990; Fukuhara et al., 1998).

The brain microvessels are formed by a specialized endothelium that regulates the interchange of solutes between the blood and the brain. Endothelial cells are coupled by both adherens and tight junctions (paracellular flux); there are also very few transcytotic vesicles in brain microvascular endothelial cells (transcellular flux). Studies report that SAH causes permeability increases in tight junctions and vesicles (Doczi, 1985; Davis et al., 1986). Two studies of SAH separately emphasize the importance of transcellular flux in the acute stage (Nakagomi et al., 1989a) and an opening in the interendothelial space in the chronic stage (Nakagomi et al., 1989b). The disruption of the BBB following SAH may be the underlying mechanism for raised ICP (Doczi, 1985), hypertension (Doczi, 1985), the toxicity of blood breakdown products, or the production of some substances such as free radicals, ET-1, arachidonic acid, and thromboxane-A2 (TXA2) (Doczi et al., 1986; Zuccarello et al., 1988).

It has been postulated that altered expression of water channels, termed aquaporins (AQPs), may contribute to edema formation after SAH. Badaut et al. (2002, 2003), using Western blot, found a marked increase in AQP1 and AQP2 protein expression 24 h after SAH in the human neocortex. Immunohistochemical studies revealed that under normal conditions, AQP4 protein was localized on astrocytic foot ends around vascular tissues. Following SAH, marked swelling of astrocytes was accompanied by diffuse AQP4 labeling, that comprised all astrocytic processes. Authors suggested that AQPs may be involved in the dynamics of brain edema formation after SAH. It has been demonstrated by others that mice knockouts for the AQP4 gene presented markedly reduced brain edema following permanent focal cerebral ischemia (Manley et al., 2000).

Relatively few studies have examined the mechanisms contributing to the development of BBB disruption after SAH. It has been suggested that blood itself and/or active substances derived as part of the degradation of vasoactive substances, such as free radicals (Imperatore et al., 2000), ET-1 (Narushima et al., 1999), arachidonic acid (Zuccarello and Anderson, 1989), and TXA2 (Zuccarello et al., 1988), present in patients with SAH, are involved in the pathogenesis of this phenomenon, as well as secondary events after SAH, such as raised ICP (Doczi, 1985) and hypertension (Doczi, 1985). Cerebral ischemia resulting from the initial bleeding promotes the accumulation of HIF-1α, which targets several adaptive genes, including glycolysis, erythropoiesis, and angiogenesis. One of the downstream factors for angiogenesis is vascular endothelial growth factor (VEGF), that is involved in the dysfunction of BBB (Mayhan, 1999; Martinez-Estrada et al., 2003). VEGF is an endothelial mitogen and is expressed in neuronal and vascular tissues in the brain (Ogunshola et al., 2002). VEGF binds with its receptor (a receptor tyrosine kinase) and activates mitogen-activated protein kinase (MAPK)-signaling pathways (Chow et al., 2001; Ogunshola et al., 2002). The levels of VEGF in the bloody CSF and the expression of VEGF in the brain tissues are both increased after SAH (Josko et al., 2001; McGirt et al., 2002; Borel et al., 2003), which might enhance BBB permeability (Mayhan, 1999; Zhang et al., 2000). Src tyrosine kinase is downstream of VEGF activation (Eliceiri et al., 1999; Irving and Bamford, 2002), and Src is activated after experimental SAH (Marton et al., 1996; Vollrath et al., 1998; Patlolla et al., 1999). Src regulates VEGF-mediated BBB permeability alteration (Eliceiri et al., 1999); inhibition of Src attenuates vascular permeability, decreases brain edema, and reduces brain infarct following ischemic stroke (Paul et al., 2001). In addition, VEGF fails to enhance vascular permeability in c-Src knockout mice (Paul et al., 2001). Thus, Src tyrosine kinase may represent a key molecular factor in early brain injury after the initial bleeding (Kusaka et al., 2004).

In an endovascular perforation model of SAH, within 24 h after the initial bleeding, VEGF and MAPK activities increased remarkably in cerebral arteries and, to a lesser degree, in the cerebral cortex adjacent to the basal cistern. These molecular changes contributed to the altered permeability of BBB, enhanced brain edema, and elevated ICP, and these pathological events led to neurological deficit and eventually death in rats (Kusaka et al., 2004). Inhibition of Src-family tyrosine kinase abolished these molecular changes, reduced BBB disruption, increased brain edema and ICP, and decreased mortality. An interesting observation was that PP1, the Src-family tyrosine kinase inhibitor, also reduced the expression of VEGF in the cerebral cortex and arteries (Kusaka et al., 2004). This new observation indicates that Src is not only downstream of VEGF receptor activation (Irving and Bamford, 2002) and leads to MAPK-signaling cascades but is also upstream of VEGF and may be involved in the signaling pathways that lead to the activation of VEGF. It needs to be noted that it is not only VEGF that stimulates Src-MAPK pathways; other growth factors, cytokines, glutamate, and free radicals also do so (Irving and Bamford, 2002).

It has been established that VEGF is a substrate of HIF-1α, which accumulates in cells after hypoxia due to cerebral ischemia, including focal cerebral ischemia (Bergeron et al., 1999), global ischemia (Chavez and LaManna, 2002), and cerebral hemorrhage (Jiang et al., 2002). The activation of HIF-1α is regulated by MAPK (Mottet et al., 2002; Sang et al., 2003). MAPK p38 is involved in HIF-1α-dependent (Gao et al., 2002) or -independent activation of VEGF (Duyndam et al., 2003). Extracellular signal-regulated kinase 1/2 (ERK1/2) MAPK activates the VEGF promoter at the proximal region under normal conditions and stabilizes HIF-1α under hypoxic conditions to enhance VEGF activation (Berra et al., 2000). Although further evidence is needed, it is possible that SAH activates Src–MAPK (Marton et al., 1996; Vollrath et al., 1998; Zubkov et al., 2001), which enhances HIF-1α-VEGF (Berra et al., 2000; Sang et al., 2003), which then activates Src–MAPK (Irving and Bamford, 2002) to contribute to early brain injury following SAH.

Some recent observations demonstrated the presence of apoptosis in the brain microvessel endothelial cells and the disruption of BBB after SAH (Zhou et al., 2004). The observed apoptotic death of endothelial cells after SAH is consistent with our previous publication, which showed that apoptosis occurred in cerebral endothelial cells in a patient who died of cerebral vasospasm after SAH (Zubkov et al., 2000a). One of the responsible agents for apoptosis may be erythrocytes or their degradation products such as oxyhemoglobin (OxyHb). It was reported that a lesser number of selective inhibitors have been identified, which inhibit the function of caspase-2, -3, -8, and -9 and effectively protect endothelial cells from OxyHb-induced apoptosis (Meguro et al., 2001b). Erythrocyte lysate causes important events, such as intracellular Ca^{2+} elevation (Zhang et al., 1996) and the generation of reactive oxygen species (Misra and Fridovich, 1972). The role of

endogenously generated reactive oxygen species in apoptosis is relatively unclear, but it has been reported that exogenously generated reactive oxygen species have led to cell death through necrosis and apoptosis (Burlacu et al., 2001). It is reported that lipid peroxidation of cell membranes causes increases in eicosanoid TXA2 (Weir et al., 1999). Stimulation of the endothelial cells with TXA2 mimetic IBOP induced apoptosis by inhibiting phosphorylation of Akt kinase, an intracellular mediator required for cell survival (Gao et al., 2000). In recent years, it has been shown that natriuretic peptides, atrial natriuretic peptides (Nelson et al., 1984), brain natriuretic peptides (Sviri et al., 2000, 2001), and C-type natriuretic peptides (Ikeda et al., 2001) were elevated in the plasma or cerebrospinal fluid of patients with SAH. An in vitro study showed that these peptides induced apoptosis via the cyclic guanosine monophosphate (cGMP)-dependent mechanism (Suenobu et al., 1999). In addition, inflammatory substances released after SAH may be in contact with microvessels and enhance vascular permeability through BBB disruption (Maruo et al., 1992; Megyeri et al., 1999; Abbott, 2000). Interleukin-1β (IL-1β), administered centrally, induced inflammation and contributed to vasogenic edema formation (Holmin and Mathiesen, 2000). IL-6, IL-1β, IL-1 receptor antagonist, and tumor necrosis factor α (TNF-α) levels all increased in bloody CSF after SAH (Mathiesen et al., 1993, 1997; Kikuchi et al., 1995; Gaetani et al., 1998; Gruber et al., 2000; Fassbender et al., 2001; Takizawa et al., 2001).

4 Apoptosis in Early Brain Injury

One of the key factors of BBB disruption and brain edema may be apoptotic cell death of neurons (Matz et al., 2000a, b, 2001) and cerebral endothelial cells (Ogihara et al., 1999; Meguro et al., 2000, 2001a, b; Kimura et al., 2003). A large family of cysteine aspartyl proteases known as caspases mediates apoptosis (Martin, 2001). The caspase family has two major subfamilies; initiators (caspase-2, -8, -9, -10) and effectors (caspase-3, -6, -7) (Martin, 2001). Among caspases, caspase-3 seems to play an essential role in cell death machinery (Lo et al., 2003).

Caspase inhibitors prevent or reduce apoptosis in a variety of tissues and reduce brain injury in animal models of cerebral ischemia (Schulz et al., 1999; Robertson et al., 2000; Loetscher et al., 2001). Antiapoptosis has been applied as a new therapy for cerebral vasospasm by protecting cerebral endothelial cells (Aoki et al., 2002; Zubkov et al., 2002) and has achieved remarkable results (Zhou et al., 2004).

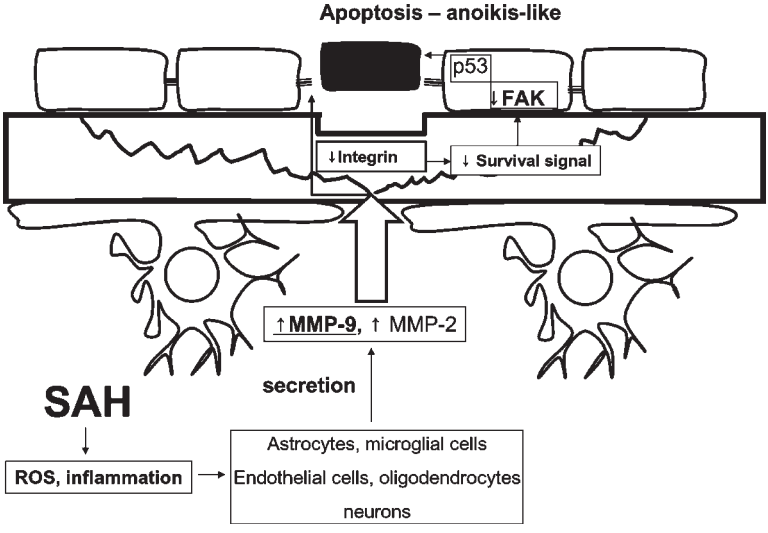
Apoptotic cell death in neuronal tissues has been reported previously after experimental SAH (Matz et al., 2000a, b, 2001). In several previous studies, Matz et al. injected lysed blood into the subarachnoid space over the dorsal aspect of the cortex beneath the coronal suture and observed 5′-triphosphate-biotin nick end-labeling (TUNEL) positive cells in the neocortex closest to the site of hemolysate injection (Matz, 2000a, b, 2001). In an endovascular perforation model, apoptotic changes were noted in most brain regions, especially in the basal cortex and hippocampus. The occurrence of apoptosis in the hippocampus is probably due to use of the endovascular filament model, which results in a drastic rise of ICP and reduction of CBF (Bederson et al., 1995; Veelken et al., 1995; Schwartz et al., 2000a), which causes global ischemia (Bederson et al., 1995; van den Bergh et al., 2002). Therefore, caspase-3 and TUNEL positive cells were most evident not only in the basal cerebral cortex, which is closest to hematoma caused by endovascular puncture, but also in CA1 and dentate gyrus regions of the hippocampus, which is known to be most vulnerable to ischemic injury (Garcia, 1988; Sharp et al., 2002).

It has been reported previously that not only neuronal cells but also cerebral endothelial cells die of apoptosis after SAH, characterized by positive TUNEL staining and double labeling of TUNEL with caspase-3 (Zhou et al., 2004). Apoptotic endothelial cell death has been studied extensively in culture conditions (Ogihara et al., 1999; Meguro et al., 2000, 2001a, b; Akin et al., 2002; Kimura et al., 2003), and we have shown that OxyHb induces apoptosis in endothelial cells in vitro (Ogihara et al., 1999; Meguro et al., 2001a, b). Endothelial apoptosis after SAH may be caused by mechanical stimulation such as elevated ICP (Bederson et al., 1995), ischemia and reperfusion injuries [especially after global ischemic response after the initial bleeding (Schwartz et al., 2000a)], toxic components of blood clots (Meguro et al., 2000, 2001a, b), activation of matrix metalloproteinase-9 (MMP-9) (Hamann et al., 1999; Dijkhuizen et al., 2002), and acute vasospasm (Bederson et al., 1998). Since caspase inhibitor Z-VAD-FMK has been shown to attenuate BBB permeability, decrease brain edema, and reduce mortality, it was postulated that Z-VAD-FMK produced

an antiapoptotic effect primarily in cerebral endothelial cells but not in neuronal tissues (Park et al., 2004). Interestingly, apoptotic endothelial death is accompanied by the activation of MMP-9 (Park et al., 2004), which plays a role in the disruption of BBB especially during hemorrhagic transformation (Hamann et al., 1999; Dijkhuizen et al., 2002) (*Figure 13-2*). Apoptosis and activation of MMP-9 contribute to the enhanced permeability of BBB and result in a mixture of vasogenic and cytotoxic brain edema after SAH (Kamiya et al., 1983; Joo and Klatzo, 1989; Kempski, 2001).

Figure 13-2

Although extravasated blood does not penetrate along the perivascular space to parenchymal vessels, acute ischemia, brain swelling, and diffusion of blood clot derived substances into the brain parenchyma constitute conditions for targeting the neurovascular unit after SAH. Brain MMP-2 and -9 activation underlies basal lamina disruption and abolition of the integrins mediated survival signal from the extracellular matrix to endothelial cells. As a result, apoptosis of endothelial cells occurs, thus providing a mechanism for further BBB damage and consecutive brain edema



5 Blood Components in Early Brain Injury

Hemoglobin and ET-1 released into the subarachnoid space are involved in the pathogenesis of cortical spreading ischemia, characterized by cortical blood flow below ischemic threshold, propagating together with neuronal and astroglial depolarization wave (Petzold et al., 2003). The presence of large amounts of hemoglobin is in a direct relationship with the availability of nitric oxide in the brain after the initial bleeding since hemoglobin has a "sink-effect" which scavenges nitric oxide (Sehba et al., 2000; Schwartz et al., 2000b). Furthermore, hemoglobin has an inhibitory effect on the Na^+/K^+-ATPase activity, which plays an important role in the mechanism of depolarization (Sadrzadeh et al., 1987). Depolarization of smooth muscle cell membranes causes the opening of voltage-dependent Ca^{2+} channels and the contraction of cerebral arteries (Zhang and Cook, 1994). Hemoglobin, depolarization, and the impairment of autoregulation after the initial bleeding all contribute to the acute vasoconstriction that enhances global ischemia, as discussed earlier.

Blood clots include not only white cells, red cells, and platelets but also other biochemical substances, such as various nucleotides released during stimulation or degradation of blood cells or brain tissues (Abbracchio and Burnstock, 1998). Nucleotides or nucleosides regulate the smooth muscle tone of cerebral

arteries and potentiate the effect of hemoglobin (Zhang et al., 1995, 1996, 1997; Sima et al., 1996, 1997; Guan et al., 1998; Aoki et al., 2001). The corresponding nucleotide receptors are upregulated in spastic cerebral arteries (Carpenter et al., 2001), and inhibition of nucleotide receptors reduces cerebral artery contraction to blood lysates (Sima et al., 1996). The possible role of nucleotides in early brain injury after the initial bleeding is not clear and warrants further investigation.

Blood clot interacts with brain tissues, CSF, and cerebral vessels, and it generates from these interactions secondary spasmogens, such as 5-HT, catecholamine, endothelin, prostaglandins, and thromboxane (Cook, 1984, 1995; Dietrich and Dacey, 2000; Zhang et al., 2003). These agents may participate in the acute vasospasm, ischemic responses, BBB rupture, and brain edema (Orz et al., 1998; Inamasu et al., 2002; Cambj-Sapunar et al., 2003) and interact with each other to potentiate their sum effects (Yakubu et al., 1994; Yakubu and Leffler, 1999).

It has been postulated that MAPK may participate in the mechanism of early brain injury induced by blood components after SAH. Although the exact mechanism of hemoglobin-induced MAPK activation remains unclear, OxyHb clearly activates MAPK in the rabbit basilar artery (Zubkov et al., 2000b).

One of the possibilities is that oxidation of OxyHb to MetHb generates free radicals (such as reactive ferryl radicals and hydroxyl radicals) that can interfere with membrane lipids and initiate lipid peroxidation and activation of phospholipase A2, thereby releasing the products of the arachidonic acid cascade (Macdonald and Weir, 1994). Most of the eicosanoids are able to activate phospholipase C (PLC), leading to the formation of inositol 1,4,5-triphosphate (IP3), which will subsequently release Ca^{2+} from internal stores. Free radicals and elevated intracellular Ca^{2+} might activate MAPK (Page and Doubell, 1996).

Another main component of hemolysate is ATP, which produces contraction by activation of G protein-coupled P2 purinergic receptors (Abbracchio and Burnstock, 1998). ATP in hemolysate might activate MAPK by activating P2 receptors (Aoki et al., 2001). Endothelin, which may be generated and released after the initial bleeding, induces contraction by activation of endothelin receptors, which are G protein-coupled receptors (GPCRs) (Zimmermann and Seifert, 1998). All four G-protein subfamilies (Gq/11, Gi/o, Gs, and G412/13) have been implicated in the activation of the MAPK cascade (Sugden and Clerk, 1997). Activation of these receptors leads to stimulation of PLC and formation of diacylglycerol (DAG) and IP3. Ca^{2+} induces smooth muscle contraction via myosin light chain phosphorylation, and protein kinase C (PKC) regulates smooth muscle contraction by phosphorylating contractile proteins such as calponin. However, DAG also stimulates PKC, which either activates Ca^{2+}-dependent tyrosine kinase (Pyk2) or directly activates Raf-1. IP3 releases Ca^{2+} from intracellular Ca^{2+} stores, and Ca^{2+} may stimulate tyrosine kinases such as Pyk2. Activation of tyrosine kinases may either phosphorylate Src (or by Shc and Grb2/SOS, cascades, activate Ras) or activate Ras directly. Ras activates Raf-1, the cellular proto-oncoprotein counterpart of viral oncogene (v-raf), which in turn activates MEK and MAPK (Sugden and Clerk, 1997). In addition, thrombin is activated in CSF after SAH. Inhibition of thrombin activity leads to amelioration of cerebral vasospasm and suppression of MAPK diphosphorylation (Tsurutani et al., 2003).

SAH elicits in human brain tissues a wide range of stress responses and results in the activation of several intracellular signaling pathways, including tyrosine kinase and its substrate MAPK (Vollrath et al., 1998; Fujikawa et al., 1999; Tibbs et al., 2000). In particular, tyrosine kinase (such as Src) and MAPK are involved in several important aspects of early brain injury after the initial bleeding, especially the rupture of BBB. Three major MAPK subgroups are the ERK, c-jun N-terminal kinase (JNK), and p38 (Zhu et al., 1999). These MAPKs are activated by various stimulants, such as growth factors including VEGF, oxidative stress, and inflammatory cytokines (Chakraborti and Chakraborti, 1998; Sugden and Clerk, 1998; Chow et al., 2001; Parker et al., 2002). GPCR agonists, activate MAPK possibly through primary activation of PKC and tyrosine kinases (Laher and Zhang, 2001; Zubkov et al., 2003). ERK1/2 plays an important role in cerebral vasospasm (Zhang, 2001; Zubkov et al., 2003). However, the role of JNK and p38 in early brain injury remains to be tested, especially since both are involved in apoptosis (Irving and Bamford, 2002), which is another feature of early brain injury after the initial bleeding (Matz et al., 2000a, b, 2001).

MAPK is activated by growth factors such as VEGF or PDGF. Growth factor receptor agonists cause dimerization of the receptors and stimulate their tyrosine kinase activities. This allows the transautophosphorylation of specific tyrosine residues in the intracellular domains of these receptors. The phosphotyrosine and its neighboring C-terminal residues are recognized by the Src-homology 2 (SH2) domains in the

adapter protein Grbs. Formation of the receptor-Grb2-SOS (exchange factor) complex promotes activation of Ras and Raf-1. In some cases, activation of Ras needs a second adapter protein, such as the Shc family. Shc binds to phosphotyrosine sequences in activated receptors and then phosphorylates a tyrosine residue in its own central domain. This phosphorylation site is recognized by Grb2 SH2 domains (Sugden and Clerk, 1997). An increasing number of GPCRs agonists have been shown to increase tyrosine phosphorylation of Shc and association of Shc with Grb2/mSOS. These agonists include angiotensin II, endothelin, thrombin, bradykinin, and carbachol (Zhang, 2001).

Another aspect of the activation of MAPK by spasmogens is the coupling mechanism between GPCRs and the MAPK cascade. Most of the blood lysate components activate GPCRs. Several GPCR agonist mechanisms can activate the MAPK cascade (Page and Doubell, 1996; Gerthoffer and Singer, 2003). There are several mechanisms for such an activation.

1. Receptor tyrosine pathway: GPCR agonists, such as endothelin or thrombin, stimulate tyrosine phosphorylation of the growth factor receptors (receptor tyrosine kinases), and the activation of the receptor tyrosine kinases then stimulates the MAPK cascade.
2. PKC and Ca^{2+}: GPCR agonists raise intracellular Ca^{2+} or activate PKC and Ca^{2+}, and PKC activates protein tyrosine kinases such as Pyk2. Pyk2 then activates Ras and MAPK.
3. Src-family tyrosine kinase pathway: Some GPCR agonists, such as platelet-activating factor, thrombin, angiotensin II, and bradykinin, activate Src-family protein tyrosine kinases as well as Shc and Raf-1 by unknown mechanisms.
4. The G protein $\beta\gamma$ subunit pathway: Thrombin, adrenaline, and acetylcholine may activate the Gi protein and cause $\beta\gamma$ dimers to link GPCRs to MAPK by the Shc-Ras-Raf-MEK pathway.
5. PI 3K pathway: Activation of Shc, Raf, and MAPK by GPCR agonists is attenuated by inhibitors of PI 3K, such as wortmannin and LY294002, indicating that the PI 3K pathway may play a role. PI 3K can be activated by both receptor tyrosine kinases or GPCR agonists and may be associated with the Src family (Laher and Zhang, 2001; Zhang, 2001).

6 Amino Acids, Peptides, and Cytokines in Early Brain Injury

Glutamate present in high concentrations in plasma and red blood cells may propagate from extravasated blood to the brain, increasing local glutamate concentration. Glutamate activates *N*-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methylisoxasole-4-propionate (AMPA) receptors on neurons as well as AMPA receptors on oligodendrocytes, contributing to white matter injury after SAH. High levels of glutamate after SAH may be also caused by diminished uptake by glial cells. Expression of glutamate transporters EAAT4 and GLAST was decreased in human neonatal SAH (Inage et al., 2000). Such glial dysfunction may lead to increased extracellular glutamate levels and contribute to excitotoxic neuronal death.

After SAH, substance P (SP) and CGRP are released from cerebrovascular sensory nerve fibers and are present in elevated concentration in CSF. They stimulate cyclooxygenase-2 (COX-2) in platelets, resulting in the production of prostanoids with preferential activation of the prostaglandin E2 (PGE2) pathway. Endothelial cells under TNF-α exposure are the second source of PGE2. PGE2α and PGF2α have damaging effects on BBB, as suggested by an in vitro study (Mark et al., 2001). Activated platelets produce TXA2, which is a potent vasoconstrictor and stimulator of platelet aggregation. The Leukotriene C4 (LTC4) pathway is activated in the cerebral cortex within the first hour following SAH (Gaetani and Lombardi, 1992). LTC4 activates endothelial cells and increases their permeability, contributing to BBB breakdown in SAH. Infiltrating neutrophils and macrophages are major producers of this compound (Kobayashi et al., 1992).

CSF eicosanoid levels are raised following SAH but not sufficiently enough to be vasoactive, *per se*, within the cerebral circulation. Rebleeding and intraventricular hemorrhage are two factors associated with a worse outcome after aneurysmal SAH. The effects of rebleeding and intraventricular hemorrhage on the CSF levels of TXB2 (TXA2 metabolite), PG6-keto F1α (prostacyclin metabolite), PGF2α, and PGE2 were

examined in patients following SAH. Intraventricular hemorrhage increased the median levels of all four eicosanoids in ventricular CSF by 2.1- to 5.1-fold. In patients who rebled, the CSF median levels of all four eicosanoids were raised up to 250-fold over the normal range. These concentrations are just sufficient for cerebrovascular and neuromodulatory effects (Pickard et al., 1994).

The level of ET-1 was increased in the bloody CSF after SAH (Kraus et al., 1991; Pluta et al., 1997; Zimmermann and Seifert, 1998). The impact of the initial bleeding and the resulting global ischemia, rather than hemoglobin, may promote the production and release of ET-1 (Pluta et al., 1997). Hemoglobin inhibited ET-1 production in cultured endothelial cells and astrocytes. Endothelin-1 is released from astrocytes, but not endothelial cells, during hypoxia and is released from the brain after transient ischemia. The increase in ET-1 levels in CSF after SAH from a ruptured intracranial aneurysm appears to be the result of cerebral ischemia rather than a reflection of the cause of cerebral vasospasm (Pluta et al., 1997). Endothelin-1 produces constriction; however, this may contribute to the acute vasospasm, reduce Na^+/K^+-ATPase activity to depolarize membrane potentials, working in concert with hemoglobin in the induction of cortical spreading ischemia (Petzold et al., 2003). Moreover, 5-HT, thromboxane, and lysophosphatidic acid (LPA) released by blood clots may stimulate ET-1 production in cultured endothelial cells (Yakubu and Leffler, 1999). In peripheral tissues, ET-1 stimulates pp60 c-Src and pp125 focal adhesion kinase activity, resulting in tyrosine phosphorylation of specific cellular proteins (Force et al., 1991; Force and Bonventre, 1998). Activation of c-Src by ET-1 might link GPCRs to the primary downstream targets of nonreceptor protein tyrosine kinases (PTK). Simonson et al. (Simonson and Dunn, 1992; Simonson and Herman, 1993) demonstrated that ET-1 caused a marked increase of the level of immunoreactive tyrosine-phosphorylated proteins, suggesting that phosphorylation of PTK is a necessary step in the mitogenic response to ET-1. Endothelin-1 activates small GTP-binding protein p21Ras and increases the p21Ras-associated PI-3K activity (Foschi et al., 1997). Activation of PKC, PTK, PI 3K, and Ras may lead to the activation of MAPK in a variety of cells (Force and Bonventre, 1998). The interrelation between ET-1 and MAPK has been studied but mostly in cerebral vascular systems (Zubkov et al., 2000c; Miao et al., 2002) or the extracellular matrix (Ogihara et al., 2000). Studies to evaluate the role of ET-1 in early brain injury after the initial bleeding, such as BBB function, brain edema, and ischemia, are warranted.

The multiple roles of cytokines in cerebral vascular inflammation after SAH were reviewed (Sercombe et al., 2002). The authors reviewed the available evidence linking these factors to the development of inflammatory lesions of the cerebral vasculature, emphasizing: (1) neurogenic inflammation due to massive release of sensory nerve neuropeptides; (2) hemoglobin from lysed erythrocytes, which creates functional lesions of endothelial and smooth muscle cells; (3) activity, expression, and metabolites of lipoxygenases, cyclooxygenases, and nitric oxide synthases (NOSs); (4) the possible role of ET-1 as a proinflammatory agent; (5) serotonin, histamine, and bradykinin, which are especially involved in BBB disruption; (6) the prothrombotic and proinflammatory action of complement and thrombin toward endothelium; (7) the multiple actions of activated platelets, including platelet-derived growth factor production; (8) the presence of perivascular and intramural macrophages and granulocytes and their interaction with adhesion molecules; and (9) the evolution, origins, and effects of proinflammatory cytokines, especially IL-1, TNF-α, and IL-6. Human and animal studies on the use of anti-inflammatory agents in SAH include superoxide and other radical scavengers, lipid peroxidation inhibitors, iron chelators, NSAIDs, glucocorticoids, and serine protease inhibitors (Sercombe et al., 2002).

In the CNS, TNF-α is cleaved from its precursor form, pro-TNF-α, by TNF-α convertase (Allan and Rothwell, 2001; Gimenez et al., 2003). A 17-kDa TNF-α acts through receptors TNFR1 and TNFR2, widely distributed in the CNS. TNF-α activates its receptor together with tumor necrosis factor receptor-associated factors (TRAF), receptor-interacting protein-1 (RIP1), and Fas-associated protein with death domain (FADD). Whereas TRAF 2/5 activate NFkB and MAPK pathways, FADD may induce apoptotic cascade through caspase-8 activation (Allan and Rothwell, 2001). In endothelial cells of rabbit basilar arteries, TNF-α increased significantly 6 h after SAH and was no different from the basal level on the third day post-SAH. It was shown that TNF-α induced apoptosis in cultured cerebral endothelial cells (via caspase-3), resulting in PARP cleavage and DNA fragmentation (Kimura et al., 2003). TNF-α, IL-1β, and IL-6 concentration were increased up to 1000-fold in CSF of SAH patients as compared with control subjects (Fassbender et al., 2001). Since TNF-α was measured at the same time as other cytokines in most studies,

similar results were obtained from numerous studies (Kikuchi et al., 1995; Gaetani et al., 1998; Gruber et al., 2000; Fassbender et al., 2001; Takizawa et al., 2001).

Elevated levels of IL-1β were found in CSF of patients after SAH (Gruber et al., 2000). It has been postulated that IL-1β may exacerbate cerebral ischemia after SAH (Fassbender et al., 2001). Moreover, intracisternal injection of IL-1β evokes COX-2 expression in cerebral arteries (Osuka et al., 1998b). CSF levels of IL-6 were increased in SAH patients (Gruber et al., 2000). Other reports demonstrated similar observations (Mathiesen et al., 1993, 1997; Suzuki et al., 1994; Kikuchi et al., 1995; Hirashima et al., 1997; Gaetani et al., 1998; Osuka et al., 1998a; Gruber et al., 2000; Fassbender et al., 2001; Nam et al., 2001; Takizawa et al., 2001).

7 Oxidative Stress

Oxidative stress is initiated by ischemia-reperfusion, which is present in the acute phase of SAH and is further aggravated by hemoglobin released into the subarachnoid space. Free radicals, triggered by hemoglobin action, include superoxide anion, hydrogen peroxide, and hydroxyl radical (Gaetani and Lombardi, 1992; Sayama et al., 1999). Superoxide anion ($O_2^{\bullet -}$) is produced by hemoglobin autooxidation. Dismutation of two $O_2^{\bullet -}$, catalyzed by superoxide dismutase (SOD), forms hydrogen peroxide (Macdonald and Weir, 1994). The latter is the source of hydroxyl radical in reactions catalyzed by a ferric ion.

The mechanism of neuronal injury triggered by free radicals involves a release of mitochondrial cytochrome *c* into the cytosol, leading to caspase-3 activation. It was shown that exposure of murine brain to subarachnoid hemolysate produced cytochrome *c*-mediated DNA fragmentation and cell death in the ipsilateral neocortex, the effects strengthened in mutant mice with a heterozygous knockout Mn-SOD gene (Matz et al., 2001). Reduction of Mn-SOD, a mitochondrial form, may result in an increased superoxide radical formation subsequently converted into hydroxyl radical in the presence of iron. In line with the aforementioned observation, hemolysate injected into transgenic mice overexpressing CuZn-SOD induced less extensive cell death than observed after injection into wild-type animals. Consistently, CuZn-SOD heterozygous knockout mutants presented a >40% increase in the cell death in the neocortex adjacent to the hemolysate injection (Matz et al., 2000a).

The pattern of enzymatic antioxidant activities in the brain cortex of rats subjected to experimental induction of SAH was studied in order to discuss the modifications of antioxidant systems in relation to the development of lipid peroxidative processes occurring in the brain cortex. The observations indicated that in the brain compartment, a significant reduction of antioxidant enzymatic activities is related to the increasing trend of enzymatic lipid peroxidation. The enhancement of enzymatic lipid peroxidation via the lipoxygenase pathway seems to play a primary role in the brain response to SAH (Gaetani and Lombardi, 1992).

It has been suggested that NOS participates in both aneurysmal formation and rupture and resultant brain injury (Fukuda et al., 2000; Sadamasa et al., 2003). Brain nitric oxide, nitrite, and nitrate levels were markedly decreased at 3 h after the initial bleeding (Sehba et al., 2000). In contrast, mRNA for iNOS was highly elevated starting from day 1 after SAH and was localized in vascular tissue but not in the brain parenchyma (Sayama et al., 1999).

Disturbances of the L-arginine-nitric oxide vasodilatory pathway have been implicated as a cause of acute vasoconstriction and ischemia after SAH (Schwartz et al., 2000b). Because nitric oxide-dependent vasodilatory mechanisms are still intact in this setting, acute vasoconstriction may be the result of limited nitric oxide availability after SAH. There is a biphasic pattern of nitric oxide availability after SAH: nitric oxide-mediated vasodilation is limited during the first 30 min of SAH and is restored 60 min after SAH (Schwartz et al., 2000b). Blood released during SAH leads to vasoconstriction by scavenging nitric oxide and limiting its availability (Sehba et al., 2000). This was tested by measuring the major nitric oxide metabolites, nitrite and nitrate, in five different brain regions before and after experimental SAH. The nitric oxide metabolites decreased significantly 10 min after SAH in all brain regions except the hippocampus. They recovered to control levels in the cerebellum at 60 min after SAH and in the brain stem and dorsal cerebral cortex 180 min after SAH; they remained low in the ventral convexity cortex. Nitrite recovered completely in all brain regions at 180 min after SAH, whereas nitrate remained decreased in the brain stem and ventral

convexity cortex. SAH causes acute decreases in cerebral nitric oxide levels by a mechanism other than NOS inhibition and provides further support for the hypothesis that alterations in the nitric oxide vasodilatory pathway contribute directly to the ischemic insult after SAH (Sehba et al., 2000). SAH causes alterations in the production or hydrolysis of cGMP or responsiveness to cGMP in the rat basilar artery in vivo. An increased rate of cGMP hydrolysis by phosphodiesterase enzyme type V (PDEV) may be a major factor contributing to the impairment of nitric oxide-mediated cerebral vasodilatation after SAH (Sobey, 2001). The mechanism of arterial vasoconstriction caused by OxyHb production after SAH was investigated. Fe^{2+}-catalyzed hydroxyl radicals generated from OxyHb-derived free radicals induced the elevation of Ca^{2+} by inhibiting the ATP-dependent Ca^{2+} pump rather than the Ca^{2+} channels in the sarcoplasmic reticulum, and it was suggested that thiols may prevent Ca^{2+} pump inactivation by inhibiting the oxidation of membrane sulfhydryl groups (Arai et al., 1999).

8 Perspectives

It is believed that knowledge on molecular signaling pathways in SAH will allow novel therapeutic approaches to inhibiting pathological signal transduction and diminishing early brain injury. The insufficiency of drugs that possess specificity and efficacy at targeting pathological signal transduction provides a great challenge for investigators in this field. Many inhibitors have remarkably adverse effects if administered at therapeutic concentrations. Alternative approaches would be to develop substances mimicking enzyme substrates to blockade enzyme-mediated signal transduction, antisense nucleotides and double stranded RNA-based gene-silencing therapeutics to diminish the expression of proteins promoting brain injury, and gene therapy to enhance cytoprotective endogenous defenses. To increase effectiveness of the above-mentioned therapies, further studies of brain molecular machinery after SAH are needed. Different approaches are suitable for therapeutic interventions interfering with highly redundant molecular mechanisms as compared to those with a single pathway involved. Therefore, implementing and testing novel molecular therapies in SAH requires extensive proteomic and genomic studies.

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14 Minocycline in Cerebral Ischemia and Excitotoxicity

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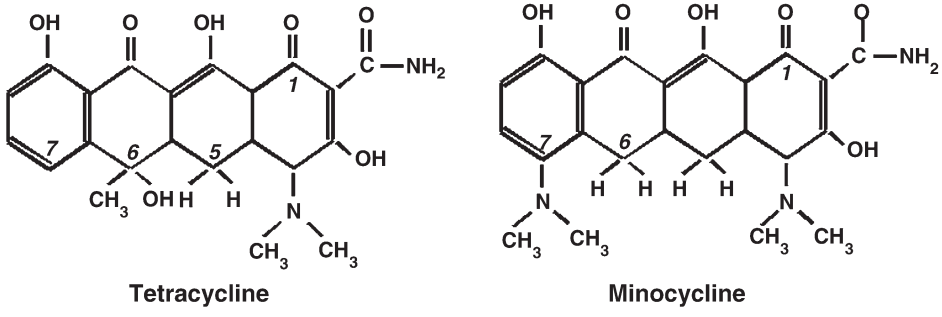
Abstract: Minocycline is a semisynthetic antibiotic of the tetracycline family that has neuroprotective and other beneficial properties in various models of neurological diseases, including cerebral ischemia and other brain diseases involving excitotoxicity. It exerts several antiinflammatory effects, which are seen as reduced microglial activation upon neuronal injury and neurodegeneration. Recent studies demonstrate that minocycline targets also several mediators of apoptotic cell death. Because minocycline has been widely used in subchronic infections for several decades, its clinical safety record and tolerability are very good. Although clinical trials with minocycline in several neurodegenerative diseases have been launched, and clinical testing of minocycline in ischemic brain diseases is being considered, some controversy exists about its efficacy and safety. The purpose of this chapter is to review the experimental data of minocycline in models relevant for ischemic brain diseases and excitotoxicity. We also review the most likely mechanism of minocycline's protective effects.

List of Abbreviations: AIF, apoptosis-inducing factor; ALS, amyotrophic lateral sclerosis; AMPA, α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid; Apaf, apoptosis-activating factor; Bcl-2, B cell lymphoma protein 2; ECM, extracellular matrix; H-I, hypoxic-ischemia; ICE, interleukin-1β-converting enzyme; MAPK, mitogen-activated protein kinase; MMP, matrix metalloprotease; NMDA, *N*-methyl-D-aspartate; TBI, traumatic brain injury; TTC, 2,3,5,-triphenyltetrazolium chloride

1 Introduction

Tetracyclines are bacteriostatic antibiotics that are active against gram-positive and gram-negative bacteria, chlamydias, mycoplasms, rickettsia, and even amoebas, by inhibiting their protein synthesis (Sande and Mandell, 1985; Sum et al., 1998) (*Figure 14-1*). These drugs are thought to bind 30S subunits of ribosomes where they prevent elongation of the developing polypeptide chains by blocking the contact

Figure 14-1
The molecular structure of tetracycline and its semisynthetic derivative minocycline. One should note the difference in substitute groups of carbons 6 and 7



between aminoacyl–tRNA and the mRNA–ribosome complex (Schnappinger and Hillen, 1996; Auerbach et al., 2002). After the first tetracycline was isolated in 1953, several derivatives were synthesized including minocycline in 1972. Today, minocycline is a widely used antibiotic for long-lasting infections. For example, in the UK, over 6.5 million people receive in average 9-month treatment with it. Minocycline is also used at the dose of 100–200 mg/day for sexually transmitted diseases and even rheumatoid arthritis (Good and Hussey, 2003). It is well absorbed by gastrointestinal tract even in aged population (Klein and Cunha, 1995). The half-life of minocycline is 11–33 h (Aronson, 1980). The lipid solubility of minocycline is tenfold better than that of tetracycline, making the drug useful also for diseases affecting the central nervous system (CNS) (Cunha and Carabedian-Ruffalo, 1990). Overall, minocycline is considered a safe drug in clinics, and only in 1.6 cases per million exposures severe adverse reactions, including lupus, hypersensitivity syndrome,

vestibular disorders, or serum sickness-like reaction may develop (Goulden et al., 1996; Shapiro et al., 1997; Schlienger et al., 2000; Settgast et al., 2003).

Minocycline and some other tetracycline derivatives have antiinflammatory properties. Initially, Golub et al. (1984) reported that minocycline inhibits collagenases and soon it was found to depress release of oxygen radicals from leukocytes (Gabler and Creamer, 1991; Gabler et al., 1992), scavenge superoxide and peroxynitrate (Whiteman and Halliwell, 1997), and even inhibit inducible nitric oxide synthase in macrophage-like or other nonneuronal cells (Amin et al., 1996). Importantly, Clark et al. (1994) reported that in rabbits, average length of reversible spinal cord ischemia required to produce paraplegia was decreased after doxycycline treatment, giving a hint that minocycline might be efficient in CNS injury and neurodegenerative diseases.

In 1998, we reported for the first time that minocycline is neuroprotective in global ischemia model (Yrjanheikki et al., 1998). Since then, minocycline has been reported to have beneficial effects in a number of neurological diseases or conditions (*Table 14-1*). Here, we review the current state in research concerning minocycline as a promising therapeutic agent in ischemic brain diseases.

Table 14-1

Animal models of neurological diseases or conditions in which minocycline acts as a neuroprotectant

Model	Source
Ischemic stroke (focal ischemia, transient)	Yrjanheikki et al. (1999), Xu et al. (2004)
Ischemic stroke (focal ischemia, permanent)	Koistinaho et al. (2004)
Embolic stroke (focal ischemia, clot model)	Wang et al. (2002, 2003a)
Hemorrhagic stroke	Power et al. (2003)
Cardiac arrest (global ischemia)	Yrjanheikki et al. (1998)
Neonatal asphyxia (postnatal H-I)	Arvin et al. (2002)
Neonatal asphyxia (postnatal excitotoxicity)	Dommergues et al. (2003)
Head trauma (TBI)	Sanchez Mejia et al. (2001)
Spinal cord injury	Lee et al. (2003), Wells et al. (2003), Stirling et al. (2004), Teng et al. (2004), Kerr and Patterson (2004)
Retina injury	Babtiste et al. (2004), Hughes et al. (2004), Zang et al. (2004)
Multiple sclerosis	Brundula et al. (2002), Popovic et al. (2002), Metz et al. (2004)
Amyotrophic lateral sclerosis	Tikka et al. (2002), Zhu et al. (2002), Kriz et al. (2002, 2003), van den Bosch et al. (2002), Zhang et al. (2003), Nguyen et al. (2003), Darman et al. (2004)
Parkinson's disease	He et al. (2001), Du et al. (2001), Wu et al. (2002), Tomas-Camardiel et al. (2004)
Huntington's disease	Chen et al. (2000), Wang et al. (2003b)
HIV	Si et al. (2004)
Alzheimer's disease	Hunter et al. (2004), Ryu et al. (2004), Wang et al. (2004b)

2 Neuroprotection by Minocycline Treatment

Minocycline has been reported to provide significant protection against ischemic, hemorrhagic, hypoxic, and traumatic neuronal injury (*Table 14-1*). Although there are a large body of evidence for the beneficial effect of minocycline, some studies have indicated that minocycline may worsen the outcome

in some models of neurodegenerative diseases (Diguet et al., 2003, 2004; Smith et al., 2003; Yang et al., 2003) and even in the mouse model of hypoxic-ischemic (H-I) brain injury in a neonatal mouse model (Tsuji et al., 2004).

2.1 Minocycline in Animal Models of Adult Brain Ischemia

The consequences of cardiac arrest or cardiac injury include transient but severe global ischemia in humans, which can be experimentally modeled by occlusion of the common carotid arteries in gerbils. The most ischemia-sensitive neurons in the gerbil model of forebrain ischemia are in layer II/III of the parietofrontal cortex and pyramidal neurons in the CA1 section of the hippocampus (Hwang et al., 2004). In the hippocampus, the neuronal death is not detected until 3–5 days after induction of the ischemia.

In our gerbil model of global brain ischemia, a 6-min occlusion of carotid arteries resulted in the loss of 89.5% of the CA1 pyramidal neurons by day 6 (Yrjanheikki et al., 1998). When the gerbils were injected with 45 mg/kg of minocycline 12 h before ischemia, and thereafter at a dose of 90 mg/kg twice a day during the first day after ischemia and 45 mg/kg starting 36 h after ischemia, the loss of CA1 pyramidal neurons was reduced to 23.3%. When the treatment was started with the dose of 90 mg/kg 30 min after ischemia, neuroprotection was comparable (28.6% of neurons lost) to that with the pretreatment protocol. Doxycycline with similar dosing was also significantly neuroprotective, reducing the death of CA1 pyramidal cells to 42.8% and 52.9%, after pretreatment and posttreatment protocols, respectively. Tetracycline hydrochloride did not provide protection. The treatments did not reduce the postoperative body temperatives. To our knowledge, no other studies on the effect of minocycline in global ischemia models have been published. Because minocycline was more neuroprotective than doxycycline in our global ischemia model, most of the later studies with tetracyclines and brain diseases have concentrated on minocycline.

We next investigated the effects of minocycline in focal brain ischemia (Yrjanheikki et al., 1999). Using the intraluminal thread model (Koizumi et al., 1986) in Sprague-Dawley rats, we tested the effect of minocycline with both pretreatment and posttreatment protocols on 90-min ischemia followed by reperfusion for 3 days. The rats were given 45 mg/kg intraperitoneally (i.p.) twice for the first day and 22.5 mg/kg for the subsequent 2 days. At these doses, minocycline did not alter rectal temperature, arterial blood pressure, plasma glucose, or arterial blood gases. When the treatment was started 12 h before ischemia, the size of the infarct was reduced by 76% in the cortex and by 39% in the striatum. A less dramatic but still statistically significant reduction in the size of both cortical (by 65%) and striatal (by 42%) infarcts was observed, when minocycline administration was started 2 h after the ischemic insult. Finally, the cortical infarcts were 63% smaller even when the treatment was started 4 h after the onset of ischemia, indicating a wide therapeutic time window for minocycline treatment in transient focal ischemia.

Xu et al. (2004) used the same ischemia model of Sprague-Dawley rats with 90-min ischemia followed by reperfusion for 1 day (24 h) but administered minocycline intravenously three times 3 (9 mg/kg in total) or 10 mg/kg (30 mg/kg in total) during a period of 8 h and starting the treatment at 4, 5, or 6 h after the onset of ischemia. They observed that at the dose of 3 mg/kg, minocycline reduced the infarct size by 42% when administered 4 h after the onset of ischemia and by 34% even when administered at 5 h. At the dose of 10 mg/kg, the protection percentages for corresponding administration time points were a bit higher, 56% and 40%, respectively. Administration at 6 h did not provide any significant protection. These studies indicate that relatively low doses, corresponding to the standard 200 mg/day dosing in humans, result in significant protection with a reasonable time window, when minocycline is administered intravenously (Fagan et al., 2004).

Wang et al. (2002, 2003a) studied the neuroprotective potential of minocycline in an embolic model of stroke in which preformed clot was introduced to vicinity of the origin of the middle cerebral artery. Minocycline was administered as by Yrjanheikki et al. (1999) in the suture model starting the treatment 1 h after the onset of ischemia. At 48 h the infarct volume was reduced by 42% in minocycline-treated Wistar rats. In the same studies, hypothermia was observed to slightly but not significantly reduce (14%) the size of clot-induced infarcts but combination of hypothermia with minocycline did not add to the protection achieved with minocycline alone.

Because a majority of the occlusions of the middle cerebral artery in humans is not associated with reperfusion during the first hours, we addressed the question whether minocycline rescues brain tissue also in permanent ischemia. We used several mouse strains and determined the infarct sizes either with a traditional histological staining (2,3,5-triphenyltetrazolium chloride, TTC) or T2-weighted magnetic resonance imaging. We administered minocycline at 12-h intervals at the dose of 60 mg/kg during the first 24 h after ischemia and thereafter at the dose of 45 mg/kg until sacrifice (Koistinaho et al., 2004). In FVB/N mice, the treatment started 12 h before ischemia reduced the infarct size by 33% at 24-h time point. When the studies were extended to other mouse strains and longer follow-up times, we found that in Balb/C mice minocycline reduced the infarct size by 28% and in C57Bl/6 mice by 50% at 3 days after ischemia, when the treatment was started prior to ischemia. Overall, despite the strain-dependent variability, minocycline was found to be beneficial also in permanent brain ischemia. However, minocycline treatment started 2 h after the onset of ischemia in Balb/C mice reduced the infarct size only by 11%, which was not statistically significant. Because aminoguanidine, an inhibitor of inducible nitric oxide synthase, provided a 33% protection when given 24 h after the permanent ischemia in Balb/C mice, the negative results with posttreatment protocol of minocycline were not due to overwhelming infarction or severity of the model. Although we did not test the effect of higher doses or intravenous administration route, the results indicate that minocycline is much less promising, if at all, in brain ischemia which is not followed by reperfusion.

Whether minocycline is protective in hemorrhagic stroke has not been fully explored. Power et al. (2003) applied collagenase-induced hematoma as a model of intracerebral hemorrhage. In this model glial activation, apoptosis and infiltration of monocytic cells are observed, and as expected, various matrix metalloproteases (MMP), including MMP-12, are induced. Minocycline was found to downregulate all these pathological changes, including apoptosis, and improved neuropathological outcome. These results suggest that minocycline could be a potential therapeutic agent also in hemorrhagic stroke.

2.2 Minocycline and Neonatal Brain Injury

H-I brain injury in the perinatal period often leads to encephalopathy with mental impairment, epilepsy, and cerebral palsy (Volpe, 2000; Berger et al., 2002). Therapeutically the situation with neonatal H-I is even worse than with adult stroke, as there are currently no intervention and the adverse effects of any drug may be more severe during early development.

Arvin et al. (2002) tested the effect of minocycline treatment using a rat H-I model in which the left carotid artery of 7-day-old pups is permanently ligated before exposing the pups to hypoxia (8% oxygen) for 2.5 h. During the following 7 days, this procedure leads in large brain damage, extending from the striatum and cortex to the hippocampus on the side of the occluded artery. When 45-mg/kg minocycline was administered i. p. immediately before the hypoxia period, a dramatic reduction of damaged areas in the striatum (23.3% → 6.5%), cortex (29.3% → 4.3%), and hippocampus (21% → −4.2%) was observed. Also 22.5-mg/kg minocycline was protective, but less significantly. Importantly, almost similarly dramatic protection from the H-I injury was observed when 45-mg/kg minocycline was given immediately after the hypoxia period. Administration of minocycline 3 h after completion of the H-I insult was too late for beneficial effects.

Tsuji et al. (2004) used the same H-I model of the same rats strain (Sprague-Dawley) and retested the effect of the pretreatment (45 mg/kg) protocol, which was found to be highly protective by Arvin et al. (2002). When minocycline was administered immediately before hypoxic exposure, the median scores of the lesion sizes were reduced by about 50% in the cortex, striatum, hippocampus, and thalamus, but the difference was not statistically significant. However, administration of minocycline 12 h before hypoxia significantly ameliorated injury in the striatum and thalamus. Also, when the data were pooled from these two experiments, minocycline treatment was found to significantly reduce the lesion size in all four brain regions. Although Tsuji et al. (2004) were somewhat able to replicate the previous findings reported by Arvin et al. (2002) in the rat, they found that minocycline treatment worsened the H-I lesion in C57Bl/6 mice. Significantly larger injury was seen in the striatum when multiple low [(45–23 mg/kg × 2)/day] or

high-dose [(135–68 mg/kg × 2)/day] injections of minocycline were given starting 12 h before hypoxic injury, and the scores were significantly worsened in the cortex, thalamus, and striatum even when a single 45-mg/kg injection was given 12 h before hypoxic exposure. These studies indicate the effect of minocycline, although a clinically approved antibiotic, may have unexpected effects in neonatal age, and that the effects of minocycline on neonatal H-I are species dependent.

Dommergues et al. (2003) tested the effect of minocycline on another mouse model, in which ibotenate, an excitotoxin, is injected intracerebrally into 5-day-old Swiss mice to mimic brain lesions of human brain palsy. In this model of neonatal brain injury, minocycline administered at the dose of 4.5 or 45 mg/kg in 12-h intervals significantly reduced the lesion in the cortical plate and white matter when scored 5 days later. The protection was better with the higher dose of minocycline, reaching 79% in the white matter and 48% in the cortical plate. Overall, minocycline may protect newborn brain also in the mouse. Whether the differences in the results are only model or strain dependent is currently unknown.

2.3 Minocycline and Excitotoxicity

In several neurodegenerative diseases and acute brain injury, such as stroke, activation of the *N*-methyl-D-aspartate (NMDA) receptors and AMPA/kainate receptors mediates to a large extent, the glutamate excitotoxicity and neuronal death (Dirnagl et al., 1999; Lee et al., 1999).

One neurodegenerative disease, in which excitotoxicity is likely to play a role, is amyotrophic lateral sclerosis (ALS) in which progressive loss of motor neurons eventually leads to death. In both clinical cases and numerous animal models of ALS, evidence exists that increased activation of AMPA receptors, possibly due to reduced expression of astroglial glutamate transporter, may be a contributory mechanism in motor neuron death (Lin et al., 1998; Carriedo et al., 2000; Howland et al., 2002; Dunlop et al., 2003). In studies on transgenic animal models of familial ALS, minocycline treatment offers considerable protection (Kriz et al., 2002, 2003; Van den Bosch et al., 2002; Nguyen et al., 2003; Zhang et al., 2003; Zhu et al., 2002) prolonging significantly the survival of mutant superoxide dismutase-1 expressing mice. Minocycline also protects against viral-induced motor neuron death (Darman et al., 2004) and against excitotoxicity of cerebrospinal fluid from ALS patients (Tikka et al., 2002). Considering that minocycline has a beneficial effect in models of several brain diseases, which involve excitotoxic mechanisms, it is not surprising that minocycline provides protection in brain and neuronal injuries triggered by excitotoxins. One such example was mentioned in the previous section, where dramatic protection of the newborn mouse brain against ibotenate (Dommergues et al., 2003), an NMDA- and metabotropic receptor agonist, was described. Minocycline also enhances discriminative stimuli of phencyclidine and MK-801, NMDA receptor antagonists, in rats (Munzar et al., 2002). Finally, induction of an NMDA receptor-dependent long-term potentiation is inhibited by β-amyloid and this inhibition is prevented by minocycline (Wang et al., 2004). Although these studies altogether indicate that neurotoxic pathways triggered or mediated by glutamate receptors are inhibited by minocycline, most of the mechanistic studies have demonstrated that minocycline is not an agent that directly blocks the function of any type of glutamate receptor.

2.4 Neuroprotective Mechanisms of Minocycline

More than 10 years ago, tetracycline derivatives, including minocycline, were shown in nonneuronal tissues to exert biological effects that are completely separate and distinct from their antimicrobial action. These properties were neither always specific nor exclusively demonstrated but included: inhibition of MMPs (Golub et al., 1991), inhibition of tumor-induced angiogenesis (Maragoudakis et al., 1994) or malignant cell growth (Masumori et al., 1994), prevention of bone resorption (Rifkin et al., 1994), depression of oxygen radical release from polymorphonuclear neutrophils (Gabler and Creamer, 1991; Gabler et al., 1992), inhibition of inducible nitric oxide synthase (Amin et al., 1996, 1997), and inhibition of protein

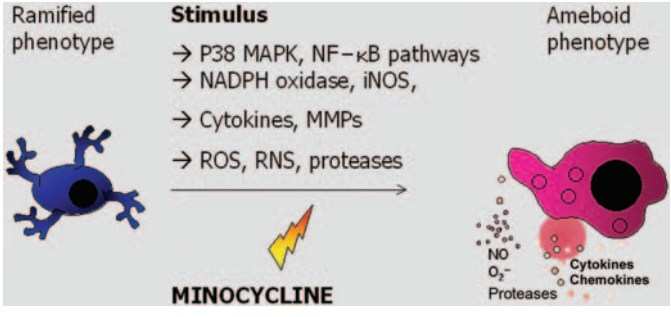
tyrosine nitration by scavenging peroxynitrate (Whiteman and Halliwell, 1997). These findings suggested altogether that minocycline has significant antiinflammatory effects. More recent studies have indicated an antiapoptotic role of minocycline, and almost all the beneficial effects of minocycline in the brain or other nervous tissue seem to be related to its inhibitory activity on inflammation or apoptotic cell death.

2.4.1 Antiinflammatory Effects of Minocycline in the Nervous System

In almost all animal experiments, the beneficial effects of minocycline have been associated with reduced microglial activation. Microglia are the major immune cells in the brain and react with phenotypical activation in response to even subtle changes in the brain homeostasis. Microglia have a key role in the innate defense of neural parenchyma against infection, ischemia, trauma, brain tumors, and neurodegeneration (Kreutzberg, 1996). Activated microglia are characterized by their ability to migrate, differentiate into amoeboid macrophage-like cells, phagocytose, and secrete a wide variety of molecules involved in inflammation, including oxygen/nitrogen radical, cytokines, and extracellular proteases (Kreutzberg, 1996; Koistinaho and Koistinaho, 2002). In the brain, neurons can also express inflammatory cytokines or enzymes such as cyclooxygenase 2 and nitric oxide synthases. On severe injury, acute inflammation is triggered by damage of the blood–brain barrier and injured blood vessels, leading to primed endothelial cells and transmigration of leukocytes (Barone and Feuerstein, 1999). These mechanisms seem to include several potential targets for minocycline, which has been reported to scavenge superoxide/peroxynitrate (as mentioned in the previous paragraphs) (Whiteman and Halliwell, 1997), inhibit expression and activity of MMP-2 and -9 (Golub et al., 1991; Paemen et al., 1996; Brundula et al., 2002; Koistinaho et al., 2004), and reduce overall leukocyte functions (Gabler and Creamer, 1991; Gabler et al., 1992). Importantly, both in vivo and in vitro data indicate that minocycline can inhibit several proinflammatory processes in microglia, such as interleukin-1β [through inhibiting expression of interleukin-converting enzyme (ICE)] (Yrjänheikki et al., 1998, 1999; Chen et al., 2000; Arvin et al., 2002) and tumor necrosis factor-α (Kim et al., 2004), inducible nitric oxide synthase (Amin et al., 1996, 1997; Yrjänheikki et al., 1998, 1999; Chen et al., 2000; Tikka et al., 2001a; Wang et al., 2003b), cyclooxygenase expression (Yrjanheikki et al., 1999; Kim et al., 2004), and NADPH oxidase (Wu et al., 2002). There is evidence that these antiinflammatory effects may be at least partially mediated by inhibition of p38 mitogen-activated protein kinase (p38 MAPK) (Du et al., 2001; Lin et al., 2001; Tikka and Koistinaho, 2001; Tikka et al., 2001a), but the direct target(s) has (have) not been yet identified (*Figure 14-2*).

Figure 14-2

The effect of minocycline on microglia. Activation and phenotypic change of microglia from ramified to amoeboid is mediated through p38/MAPK and NF-κB pathways, resulting in activation of NADPH oxidase and expression of inducible NOS, MMPs, and proinflammatory cytokines. These changes are inhibited by minocycline, most likely upstream to executioner enzymes or cytokines, targeting MAPK pathways



One important target of minocycline's neuroprotective action is expression and activity of MMPs, especially MMP-9 and -2 (Golub et al. 1991; Paemen et al., 1996; Brundula et al., 2002). Cerebral ischemia is well known to trigger extracellular protease processes in which major components of basal lamina are degraded leading to the breakdown of the blood–brain barrier and loss of microvascular integrity. (Rosenberg et al., 1998; Barone and Feuerstein, 1999; del Zoppo and Mabuchi, 2003; Fukuda et al., 2004). Vascular injury results in edema, activation of resident microglial cells, infiltration of circulating inflammatory cells into the brain and finally, neuronal death (Barone and Feuerstein, 1999; Van den Steen et al., 2002; del Zoppo and Mabuchi, 2003) Cytokine-inducible MMP, MMP-9 (gelatinase B, 92-kDa type IV collagenase) is thought to be the terminal enzyme in the ECM-remodeling cascade (Gasche et al., 1999; Van den Steen et al., 2002). Recent studies have shown that inhibition of MMP-9 by genetic, immunologic, or pharmacologic approaches reduces infarct volumes in mice (Romanic et al., 1998; Asahi et al., 2000) suggesting a deleterious role for MMP-9 in ischemic brain injury. In addition, expression of pro-MMP-2 at least in nonhuman primate models of stroke is directly related to neuronal injury and its role in ischemic injury is supported by the findings that both expression of pro-MMP-2 and its protease activity are triggered after brain ischemia (Clark et al., 1997; Chang et al., 2003; Fukuda et al., 2004).

For these reasons, we investigated the effect of minocycline in relation to MMP-9 expression using two different lines of MMP-9 deficient mice (Dubois et al., 1999; Van den Steen et al., 2002; Koistinaho et al., 2004). In wild-type control mice, minocycline treatment started before permanent occlusion of the middle cerebral artery reduced the infarct size by 50% and inhibited ischemia-provoked pro-MMP-9 induction. However, neither one of the MMP-9 deficient mouse lines had reduced lesion size when determined 3 days after the permanent occlusion of the cerebral artery. Importantly, MMP-9 deficiency blocked the infarct-reducing effect of minocycline. The possible explanation for these results resided most likely in the role of MMP-2. Pro-MMP-2 was induced by permanent ischemia in wild-type control and MMP-9 deficient mice. While ischemia-induced pro-MMP-2 was downregulated by minocycline treatment in wild-type mice, in MMP-9 knockout mice it remained at the same level as in saline-treated wild-type mice. In other words, expression of pro-MMP-2 levels were compensatorily upregulated in MMP-9 deficient mice, as suggested by previous studies (Ducharme et al., 2000) and minocycline, at least with the doses used [(60–45 mg/kg $\times$ 2)/day], was not able to downregulate them to the normal levels. These results suggest that MMP-2, but not MMP-9, may be an important target of minocycline in permanent brain ischemia. It is of interest that minocycline has been reported to inhibit p38 MAPK (Du et al., 2001; Tikka et al., 2001a), which in some models regulates MMP-2 expression (Park et al., 2002; Vayalil and Katiyar, 2004; Vitale et al., 2004) and significantly contributes to infarction after permanent occlusion of the middle cerebral artery (Koistinaho et al., 2002).

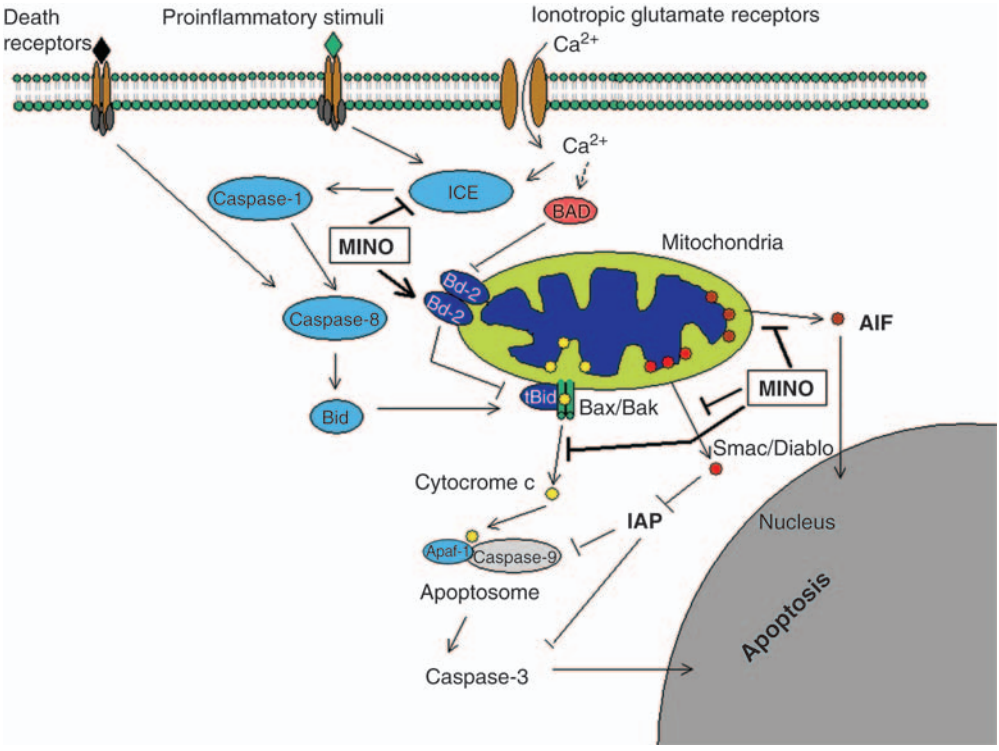
2.4.2 Antiapoptotic Effects of Minocycline

When minocycline was found to be protective in brain ischemia models, it was observed to reduce expression of ICE, a protease, which is also known as caspase-1 (Yrjanheikki et al., 1998, 1999). Subsequently, Chen et al. (2000) reported that the beneficial effect of minocycline in a transgenic R6/2 model of Huntington's disease is associated with decreased expression of both caspase-1 and caspase-3. Minocycline was also found to reduce apoptotic neuronal death in vitro both in mixed neuron–glia cultures (Tikka et al., 2001a) and in relatively pure neuronal cultures (Tikka et al., 2001b). Later on, it was shown that in models of neurodegenerative diseases minocycline inhibits the mitochondrial permeability transition-mediated cytochrome *c* release, which potently triggers activation of caspase-9 and caspase-3, thereby leading to apoptotic cell death (Zhu et al., 2002). Minocycline was also demonstrated to prevent the recruitment of apoptosis-inducing factor (AIF) and Smac/Diablo, mitochondrial mediators of caspase-independent and caspase-dependent death, respectively, in a cell culture model of Huntington' disease (Wang et al., 2003b), and evidence for similar mechanisms of minocycline's protective action was also found in cultures of neonatal and adult rat ventricular myocytes (Scarabelli et al., 2004). Still another antiapoptotic effect associated with mitochondria is upregulation of Bcl-2 (Wang et al., 2004a) in an endothelial cell model of apoptotic cell death in kidney. While it is not understood how minocycline is able to regulate so many

independent pathways and proteins of apoptotic cell death, there is ample evidence for a potent and direct antiapoptotic effect of minocycline. Whether these targets of apoptotic signaling are also relevant in neuronal death in ischemic brain injury is not yet clear (*Figure 14-3*).

Figure 14-3

Targets of minocycline in caspase-dependent and caspase-independent pathways of apoptotic cell death. Apoptosis-inducing factor (AIF); apoptosis-activating factor (Apaf); interleukin-1β-converting enzyme (caspase-1) (ICE)



3 Conclusions

The multiple targets of minocycline may explain why minocycline appears to be a potent protective agent in various models of cellular injury. On the other hand, the abundance of seemingly separate effects of minocycline may be secondary to one or a very few mechanisms of minocycline in upstream intersections of inflammatory and apoptotic pathways, which still remain uncovered. Finally, minocycline has recently been reported to display not only beneficial but also detrimental effects in different species, strains, and models of neurological disorders (Diguet et al., 2003, 2004; Smith et al., 2003; Yang et al., 2003). In brain ischemia models, no harmful effects have been observed so far, but the contradictory and possibly species-dependent results on minocycline's role in neonatal H-I warrant proceeding with caution in the clinical application of minocycline for brain diseases of ischemic and/or excitotoxic cell death.

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15 Matrix Metalloproteinases and Tissue Plasminogen Activator Reperfusion Therapy for Stroke

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Abstract: Reperfusion therapy with intravenous tissue plasminogen activator (t-PA) is the only FDA-approved Medical therapy for acute ischemic stroke. Properly titrated use of t-PA improves clinical outcomes. However, the near tenfold-associated risk of intracerebral hemorrhage after t-PA may keep this therapy from many acute stroke patients. Emerging data now suggest that some of the potentially neurotoxic side effects of t-PA may be due to its signaling actions in the neurovascular unit. Besides its intended role in clot lysis, t-PA is also an extracellular protease and signaling molecule in the brain. t-PA mediates matrix remodeling during brain development and plasticity. By interacting with the NMDA-type glutamate receptor, t-PA may amplify potentially excitotoxic calcium currents. At selected concentrations, t-PA may be vasoactive. Finally, by augmenting matrix metalloproteinase (MMP) dysregulation after stroke, t-PA may degrade extracellular matrix integrity and increase risks of neurovascular cell death, blood–brain barrier leakage, edema, and hemorrhage. Understanding these pleiotropic actions of t-PA may reveal new therapeutic opportunities for increasing the safety and efficacy of reperfusion strategies for stroke therapy.

List of Abbreviations: MMP, Matrix metalloproteinase; BBB, blood brain barrier; t-PA, tissue plasminogen activator; rt-PA, recombinant tissue plasminogen activator; NINDS, National Institute of Neurological Disorders and Stroke; ICH, intracerebral haemorrhage; HT, Hemorrhagic transformation; NVU, neurovascular unit; ECM, extracellular matrix; i.v., intravenous; CNS, central nervous system; LTP, long-term potentiation; LRP, lipoprotein receptor-related protein; PKA, protein kinase A; MCAO, middle cerebral artery occlusion; NO, nitric oxide; NOS, nitric oxide synthase; TBI, traumatic brain injury; PMNL, polymorphonuclear leukocytes; MAP kinase, mitogen-activated protein kinase; SOD, superoxide dismutase; MBP, myelin basic protein; ROS, Reactive oxygen species

1 Introduction

Stroke is a leading cause of disability in the USA, costing an estimated $40 billion per year in direct health care costs and loss of earning potential (American Heart Association, 2006). Tissue plasminogen activator (t-PA), the only FDA-approved treatment for acute ischemic stroke, is efficacious in increasing the proportion of patients free of disability at 3 months by 30%–35% and decreasing the cost of medical care for individuals who receive it (up to $4.5 million per 1,000 eligible treated patients) (Fagan et al., 1998). However, nearly a decade after the NINDS (The National Institute of Neurological Disorders and Stroke) trials, t-PA is only given to less than 5% of stroke patients. This is partly due to the tenfold increased risk of intracranial hemorrhage (ICH) in t-PA-treated patients and more than 50% mortality rate for patients with major hemorrhage (NINDS rt-PA Stroke Study Group, 1995).

Hemorrhagic transformation (HT) in ischemic stroke is heralded by damage within the neurovascular unit (NVU), which comprises the cerebral endothelial cells, astrocytes, and neurons, in a milieu of extracellular matrix (ECM) (Petty and Lo, 2002; Lo et al., 2003). Locally formed oxygen free radicals and activated plasmin lead to the secretion of proteinases in the ECM, which degrade the components of the basal lamina of the blood–brain barrier (BBB), causing vasogenic edema and extravasation of blood into the brain parenchyma (Gasche et al., 2001; Lo et al., 2004).

Matrix metalloproteinases (MMPs), the largest class of human proteases, have been implicated in both animal and human studies of t-PA-related HT. Although the benefit of intravenous (i.v.) t-PA is intravascular clot lysis, in vitro studies suggest that exogenous t-PA may exacerbate BBB breakdown by contributing to MMP activation, which mediates t-PA's extravascular adverse effects.

In this chapter, we review the clinical relevance of t-PA as a thrombolysis agent and the proposed mechanistic role of MMPs in t-PA-related adverse effects, in both in vivo and in vitro settings.

2 t-PA Trials in Acute Stroke: Clinical Significance

There have been several large randomized trials using i.v. t-PA in acute ischemic stroke. *Table 15-1* lists details of some of the major thrombolysis trials and their findings. In particular, the NINDS trial and a

Table 15-1

Major randomized thrombolysis trials. Alteplase (recombinant tissue plasminogen activator) trials underlined

Trial name	N	Dose	Time	Clinical outcome	Comments (Hemorrhage /Mortality)
NINDS (National Institute of Neurological Disorders and Stroke, NEJM, 1995)	624	IV alteplase (0.9 mg/kg up to 90 mg)	<3 hrs	12 m 30% increase in minimal to no disability; 3 m mortality NS	6% vs. 0.6% <1% severe systemic
ECASS (European Cooperative Acute Stroke Study, JAMA 1995)	620	IV alteplase (1.1 mg/kg up to 100 mg)	<6 hrs	3 m increase mortality in tx group (22% vs 16%); 3 m functional status NS (but reanalysis after excluding protocol violators w/marginally significant improvement, p = 0.04)	109/620 pts had extensive pre-tx CT abnormalities violating inclusion criterion
ECASS II (Lancet 1998)	800	IV alteplase (0.9 mg/kg up to 90 mg)	<6 hrs	90-day mortality or outcome NS	
ATLANTIS (JAMA 1999; Stroke 2003)	547	IV alteplase (0.9 mg/kg)	3–5 hr (61 pts <3 hrs)	90 day fxn outcome NS; 90 day increased mortality (11 vs 7%; p = 0.09); Subgroup analysis (tx <3 hr vs 3–5 hrs) show 35% increased favorable outcome	First 10 days increased ICH (7% vs 1%; p < 0.001)
MAST-E (Multicenter Acute Stroke Trial-Europe) France and UK	310	IV streptokinase (1.5 million units over 1 hr)	<6 hrs	10 day increased mortality in SK-tx group; 3 m (45% vs. 34%; p = 0.06); 6 m death or disability NS	Stopped early due to early mortality rate increase
MAST-I (Italy) (Lancet, 1995)	622	IV streptokinase vs 300 mg/day ASA x 10 days; vs both	<6 hrs	6 m increased mortality in SK and ASA pts (but lower rate of disability); Combined endpoint of death/disability NS	Stopped early due to 6 m increased mortality
ASK (Australian Streptokinase Lancet 1995; JAMA 1996)	340	IV streptokinase (1.5 million units over 1 hr)	<4 hrs	90-day increased mortality in tx (43% vs 22%)	Stopped early due to higher mortality; post-hoc analysis w/increased risk of adverse outcome >3 hrs

reanalysis of the ATLANTIS trial for patients given t-PA at less than 3 h have shown the benefits of i.v. t-PA in improving functional outcome. To date, i.v. t-PA is the only FDA-approved medical treatment for acute ischemic stroke. However, i.v. t-PA increases the rate of intracerebral hemorrhage (ICH) by up to tenfold NINDS rt-PA Stroke Study, 1995).

The rates of ICH vary depending on the study and protocol adherence. In community-based studies, the rates of ICH range from 3.3% to 15.7% (Katzan et al., 2000; Hill and Buchan, 2001; Albers et al., 2002). In clinical trials of i.v. alteplase (rt-PA), the rate of HT has been approximately 6% (Tanne et al., 1999; The NINDS Stroke Program Review Group, 2000; Hacke et al., 2004). Although ICH is a dreaded consequence carrying a high mortality rate, the benefit of early reperfusion by t-PA still outweighs the risks. The meta-analysis of three major clinical trials (NINDS, ATLANTIS, and ECASS) showed a relative benefit even at 4.5 h poststroke (Donnan et al., 1995; Hommel et al., 1995; Clark et al., 1999) (*Table 15-2*).

Table 15-2
Pooled odds ratio of 3 months favorable outcome from the meta-analysis of NINDS, ATLANTIS, and ECASS trials (Hacke et al., 2004)

0–90 min	2.8	1.8–9.5
91–180 min	1.6	1.1–2.2
181–270 min	1.4	1.1–1.9
271–360 min	1.2	0.9–1.5

The NINDS trial, using a narrow time window of 3 h, has been the only trial to demonstrate a 30% improvement in outcome at 12 months, one of the primary endpoints. The ECASS, ECASS II, and ATLANTIS trials all included patients within wider time windows, up to 6 h (Hacke et al., 1998b; Clark et al., 1999). ATLANTIS, after excluding protocol violators for subgroup analysis, found a significant ($p = 0.04$) improvement in functional status at 3 months (Albers et al., 2002). Subgroup analysis of ECASS for t-PA treatment under 3 h and post hoc analysis for all patients with dichotomized or global endpoints also support the use of t-PA. However, in these analyses the sample sizes become smaller and some of the endpoints were not prespecified (Hacke et al., 1998a, b; Steiner et al., 1998). Other major thrombolysis studies, involving streptokinase, have all been stopped early due to increased mortality or adverse outcome (1993; Hommel et al., 1995; Hacke et al., 2004).

The results of these trials, and of studies in vivo and in vitro, guided the current clinical practice of administering i.v. t-PA within 3 h of symptom onset. However, a better understanding of the mechanism of t-PA-related injury may lead to new and improved treatment for acute stroke. The following sections review the mechanism of t-PA-related injuries.

3 Mechanisms of t-PA-Related Neurotoxicity

3.1 How Does t-PA Work?

Endogenous t-PA, a serine protease, can be produced by the brain and is found in glial and neuronal cells (Krystosek and Seeds, 1984; Sappino et al., 1993; Chen et al., 1995; Nishibori et al., 1995; Backstrom et al., 1996; Gschwend et al., 1997; Davies et al., 1998; Scarisbrick et al., 2001; Siao et al., 2003). t-PA converts plasminogen to plasmin to provide clot lysis and fibrinolysis (Krystosek and Seeds, 1981). Endogenous t-PA is an important proteolytic force in the central nervous system (CNS), with roles not only in fibrin degradation and clot lysis but also in synaptic plasticity and transmission (Baranes et al., 1998; Hoffman et al., 1998; Neuhoff et al., 1999; Gingrich et al., 2000).

In the normal CNS, t-PA is a very versatile protease. It is important in synaptic remodeling (Baranes et al., 1998; Neuhoff et al., 1999; Wu et al., 2000) and participates in neuronal migration and growth during

learning and development (Krystosek and Seeds, 1981; Friedman and Seeds, 1995). t-PA has been actively studied in memory and learning through its mechanism in facilitating long-term potentiation (LTP) (Frey et al., 1996; Huang et al., 1996; Baranes et al., 1998; Madani et al., 1999) and long-term depression (LTD) (Calabresi et al., 2000). In pathological conditions, t-PA participates in excitotoxic injury, Wallerian degeneration, and peripheral nerve injury (Bignami et al., 1982; Tsirka et al., 1995; Chen and Strickland, 1997; Wang et al., 1998; Akassoglou et al., 2000). However, t-PA also plays a role in the body's functional recovery process such that t-PA knockout mice have delayed sensory response after damage to the sciatic nerve (Siconolfi and Seeds, 2001).

The importance of t-PA in the brain is supported by the observation that t-PA is found in multiple areas of the brain. Specifically, t-PA has been localized to all neuronal layers of the hippocampus, the meninges, vascular tissue, and neutrophil of the central nucleus and the hypothalamus (Qian et al., 1993; Sappino et al., 1993; Carroll et al., 1994; Tsirka, Gualandris et al., 1995; Salles and Strickland, 2002). In the hippocampus of mice, t-PA protein and activity have been demonstrated in the mossy fiber pathway under basal condition (Salles and Strickland, 2002). t-PA is upregulated in the CA1 neuronal cell bodies after unilateral hippocampal injection of excitatory glutamate analog, kainic acid (KA). In the perforant pathway, t-PA was identified as an immediate-early gene after high-frequency stimulation and the induction of seizures (Qian et al., 1993).

The mechanism by which t-PA participates in normal physiologic functions, such as LTP, may be related to its ability to degrade the ECM and release adhesion molecules in the formation and elongation of axons (Baranes et al., 1998; Hoffman et al., 1998). The proteolytic function of t-PA in the ECM is related to another important class of proteases, MMPs. The t-PA-MMP axis will be reviewed in detail in Sect. 8.4 (Lo et al., 2003, 2004).

t-PA-induced proteolytic activity may play a major role in the NMDA-independent mossy fiber LTP (Salles and Strickland, 2002). This was demonstrated by experiments showing that the overexpression or addition of t-PA increased and prolonged late-phase LTP (Baranes et al., 1998; Madani et al., 1999). Conversely, t-PA inhibition or knockout t-PA models had inhibition of late-phase LTP (Baranes et al., 1998). T-PA can also modify LTP by increasing the intracellular Ca^{2+} influx by cleaving the NR1 subunit of the NMDA receptor (Nicole et al., 2001; Fernandez-Monreal et al., 2004). In addition, in last-phase LTP, t-PA binds to lipoprotein receptor-related protein (LRP) and activates protein kinase A (PKA) (Zhuo et al., 2000).

Under physiologic conditions, t-PA mRNA is continually present, but its expression into protein may be under translational control such that injury causes upregulation (Salles and Strickland, 2002). After synthesis, t-PA is placed in vesicles in preparation for release. t-PA is liberated into the ECM via a calcium-dependent pathway following membrane depolarization (Gualandris et al., 1996; Parmer et al., 1997). This process is also mediated by NMDA receptor signaling (Nicole et al., 2001; Fernandez-Monreal et al., 2004). The release of t-PA into the ECM triggers a positive feedback mechanism that upregulates its mRNA levels. This process is integral to some of the pathophysiologic roles of t-PA in cerebral ischemia, which involves proteases in the ECM such as MMPs. However, t-PA is under tight regulation by various inhibitors such as type 1 plasminogen activator inhibitor (PAI-1), neuroserpin, and astrocytic receptor-dependent scavenging (Gautier et al., 2003; Fernandez-Monreal et al., 2004). These inhibitors have been investigated as neuroprotection strategies to minimize t-PA's adverse effects. As a serine protease, t-PA is also temperature sensitive, and hypothermia has been studied as another strategy in neuroprotection (Yenari et al., 1995; Maier et al., 1998).

The thrombolytic action of endogenous t-PA protects the brain from ischemic insults, as evident in t-PA-deficient mice, where fibrin deposition correlates with brain injury (Tabrizi et al., 1999). Exogenous i.v. rt-PA (recombinant t-PA) is commercially available for capitalizing on its thrombolysis function in cerebral reperfusion in ischemic brain injury and myocardial infarction. With t-PA's half-life of less than 5 min, t-PA (such as alteplase) related hemorrhagic conversion usually occurs within 24 h of treatment. While the target of t-PA is within the vessel, in the context of weakened vessels and injured brain parenchyma, t-PA's adverse effects may be related to its role outside the vessel (Lo et al., 2004). The in vivo and in vitro bases of the mechanisms of t-PA neurotoxicity are reviewed in the next section.

3.2 Animal Studies of t-PA

Animal studies on the effects of t-PA in stroke have resulted in findings ranging from neurotoxic to beneficial, depending largely on the duration of ischemia and the model and mechanism of injury (Kaur et al., 2004). *Table 15-3* below summarizes a limited number of experiments showing both sides of the story (Kaur et al., 2004).

Table 15-3
Animal models

Species	Time postinjury	Model of injury	Dose of t-PA	Conclusion	Reference
Rat WT		NMDA induced	3.0 μg	Deleterious	Nicole et al. (2001)
Mouse t-PA −/− mouse WT		Kainate induced t-PA tx	Not treated 120 ug for 3 days	Deleterious Deleterious	Tsirka et al. (1996)
Rat WT		Global	10 mg/kg	No effect	Klein et al. (1999)
Mouse WT	1.5–3 h	Transient focal MCAO	0.1, 2.0, and 10 mg/kg,	Deleterious (1.5 h; 2 mg/kg); No effect at other doses	Kilic et al. (2001)
Mouse t-PA −/− Mouse WT	2 h, 3 h	Transient focal MCAO	1.0 mg/kg	Deleterious	Wang et al. (1998)
Rat WT	1.5 h	Transient versus Permanent focal MCAO	10 mg/kg	Transient-no effect; Permanent-deleterious	Meng et al. (1999)
Rat WT		Transient focal MCAO	10 mg/kg	No effect	Klein et al. (1999)
Rat WT	72 h	Permanent MCAO	Neuroserpin, (t-PA inhibition)	Beneficial	Yepes et al. (2002)
Mouse t-PA −/−	3 h	Transient focal MCAO	Not treated	Deleterious	Tabrizi et al. (1999)
Mouse t-PA −/−		Permanent MCAO	Not treated	Deleterious	Nagai et al. (1999a)
SHR		Transient focal MCAO	3, 10, 30 mg/kg	Deleterious	Gautier et al. (2003)
Mouse t-PA −/− Mouse WT		Photothrombotic	Not treated	No effect Deleterious	Zhao et al. (2004)
Rabbit WT	1–2 h	Transient embolic MCAO	2.0 mg/kg	Beneficial	Carter et al. (1992)
Rabbit WT		Transient embolic MCAO	3.3 mg/kg	Beneficial	Lapchak et al. (2000)
Rat WT		Transient embolic MCAO	5.0 mg/kg	Beneficial	Andersen et al. (1999)
Rat WT	2 h	Transient embolic MCAO	1.5 mg/kg (TNK)	Beneficial	Zhang et al. (2000)

Adapted from Kaur et al. (2004) with permission

Studies observed a trend in the reduction of infarct volume in rt-PA-treated animals during ischemia. This dose-independent effect suggests the beneficial effects of early administration of rt-PA, presumably due to the improvement in microvasculature (Kilic et al., 2001; Kaur et al., 2004). In this section, however, we concentrate on the mechanism of t-PA-related injury.

The conflicting results of studies of t-PA-related injury may be related in part to the duration of cerebral ischemia. One study has shown that damage from mild photothrombotic chemical middle cerebral artery occlusion (MCAO) was correlated to more extensive injury in t-PA knockouts, but after severe damage t-PA knockouts had less injury (Nagai et al., 2002). It was postulated that perhaps the thrombolysis effect of t-PA is protective after mild injury, but adverse effects of t-PA obliterate this benefit in large areas of damage. In support of this theory, in the focal clip model, permanent ischemia was associated with t-PA-induced increases in the volume of infarction but transient ischemia was not (Kaur, Buchan et al., 2004).

t-PA knockout (t-PA−/−) mice have offered much insight to the study of t-PA toxicity. In the focal ischemia model in t-PA knockout mice, the administration of t-PA increased the infarct size 2 and 3 h postischemia. The t-PA knockout mice had 50% decreased volume of infarct compared to wild-type (WT) mice (Wang et al., 1998). In WT mice, there is a "ceiling effect," where t-PA induced increase in infarct size after 2 h of ischemic insult, but this effect was attenuated after 3 h (Wang et al., 1998). In models of excitotoxicity induced by unilateral intrahippocampal injection of KA, t-PA knockout mice are resistant to excitotoxic neuronal death (Carmeliet et al., 1994; Tsirka et al., 1996). However, another study showed that removal of t-PA expression did not protect against neuronal degeneration (Mecenas et al., 1997). Even at supraphysiologic doses (ten × more than human dose), t-PA administration immediately after mechanically induced global and focal transient ischemia did not result in increases in infarct volume or cytotoxic-associated CA1 selective neuronal injury (Klein et al., 1999). These differences speak to the complexity of t-PA-related adverse effects; the mechanisms of which are just unfolding.

The neurotoxicity of t-PA is thought to be partly mediated through its thrombolytic effect. In t-PA gene inactivation experiments in a mouse model of transient focal ischemia, the augmentation of t-PA activity (via PAI-I inactivation) and the reduction of plasminogen activity (via plasminogen inactivation) increased infarct size (Nagai et al., 1999a). In support of t-PA's neurotoxicity via thrombolysis, hamster models of focal ischemia using other thrombolytic agents involving plasmin activation, such as streptokinase and staphylokinase, have shown a dose-related effect in augmenting infarct size (Nagai et al., 1999b). In addition, plasminogen-deficient mice have severe thromboses, but they are resistant to excitotoxic death (Bugge et al., 1995; Tsirka et al., 1997).

t-PA also mediates excitotoxic effects via plasminogen-independent pathways. In kainate-induced seizures, t-PA knockout mice had attenuated seizure propagation, whereas plasminogen knockout mice did not have such an effect (Yepes et al., 2002). This seizure model, created by the injection of *kainate*, a glutamate analog, into the amygdala, upregulated t-PA in both the amygdala and the hippocampus and resulted in neuronal death in the hippocampus. Reserpine, a t-PA inhibitor, attenuated seizures in this model. This plasminogen-independent effect of t-PA could be a consequence of t-PA-mediated cleavage of the NR1 subunit of the NMDA receptor, which may lead to increased neuronal excitatory activity and ultimately seizures (Pawlak and Strickland, 2002).

In cell culture experiments, microglia cells from t-PA knockout mice restore microglial activation after infusion of either catalytically active or inactive t-PA prior to KA injection, demonstrating the nonproteolytic cytokine function of t-PA (Rogove et al., 1999). Subsequent studies showed that t-PA exhibits a nonproteolytic cytokine function through its finger domain binding to annexin II on the microglial cell surface (Siao and Tsirka, 2002).

Nitric oxide (NO) has also been linked to t-PA toxicity. In a mouse model of MCAO, deficiency in neuronal nitric oxide synthase (NOS) resulted in smaller infarct size (Huang et al., 1994). In other organs, such as the kidney, it is thought that NO may reduce t-PA mRNA levels and function as a modulator of gene expression (Beck et al., 1999; Eberhardt et al., 2002). Animal models of permanent focal ischemia suggested that the t-PA carrier, containing a large amount of L-arginine, a substrate for all isoforms of NOS, could act through the NMDA receptor and enhance the adverse effect of t-PA (Buchan; Warren, 2003).

The model of t-PA neurotoxicity via NMDA receptors arises from the observation that t-PA increases neuronal cell death by glutamatergic receptors. While injection of t-PA alone into the stratum was not toxic,

t-PA appears to potentiate NMDA activation since coinjection of t-PA with NMDA increased NMDA-induced lesions by 50% (Nicole et al., 2001). Although the exact mechanism is controversial, this effect was thought to be due to t-PA's proteolytic cleavage of 15- to 20-kDa fragments from the NR1 subunit of the NMDA receptor, thus amplifying the NMDA-induced increase in intracellular calcium and provoking cell death (Nicole et al., 2001). Other studies also reported the NR2 subunit to be important, suggesting that t-PA may act through multiple pathways in potentiating NMDA signaling in neuronal death (Yang et al., 2003).

In other animal models, such as the traumatic brain injury (TBI) model, t-PA knockout mice also had reduced cortical lesions and amelioration in edema volumes (Mori et al., 2001). The study of the Lurcher mutant mouse also supports the contribution of glutamate receptors to t-PA neurotoxicity through apoptotic pathways. In the Lurcher mutant mouse, a mutation in the glutamate receptor subunit gene results in aberrant inward calcium current; this in turn induces cerebellar premature apoptosis (Lu and Tsirka, 2002). In this model, the elimination of t-PA offers neuroprotection in delaying the apoptotic death of Purkinje and granule neurons.

Studies of HT in rats noted polymorphonuclear leukocytes (PMNL, aka neutrophils) accumulation in the microvessels within the ischemic core tissue (Kano et al., 2000). Late administration of t-PA (6 h) in this thromboembolic rat model had a 50% chance of developing confluent hemorrhagic infarction. Interestingly, PMNLs are a source of MMPs, which have been linked to t-PA-related hemorrhages in both in vitro and in vivo studies (Lo et al., 2003). It has been shown that neutrophil infiltration contributes to enhanced MMP-9 in the ischemic brain by releasing MMP-9 proform, which might participate in inflammatory tissue reaction (Justicia et al., 2003). However, a quantitative delineation of the sources of dysregulated MMPs remains to be fully dissected; multiple sources may dominate depending on model conditions. A recent study by Maier et al. (2004) found that in SOD1-deficient mice, neutrophils are unlikely to be a major source of MMP-9, highlighting the importance of MMP-9 production by other sources. If damaged brain is indeed the primary source of MMP elevation, MMP-9 may serve as a peripheral plasma marker of brain injury.

The next section will review the importance of the t-PA–MMP axis in the context of the NVU, a unifying conceptual model employing an integrative approach toward stroke (Lo et al., 2004).

4 The Neurovascular Unit and the t-PA–MMP Axis

4.1 The Concept of the Neurovascular Unit

In the last decade, the study of neurovascular disease was largely "neurocentric," focusing on neuronal death. Recently, the NINDS Stroke Program Review Group recommended, for the next decade, shifting from a neuro-centric research perspective to a more integrative approach incorporating the concept of the NVU, composed of cerebral endothelial cells, astrocytes, and neurons in a milieu of ECM (The NINDS Stroke Program Review Group, 2000; Lo et al., 2003, 2004) (*Figure 15-1*). This framework provides a broader understanding of the overall tissue response to stroke and its treatments through cell–matrix interactions and cell–cell signaling.

While the intravascular clot-busting effect of t-PA is the foundation of its efficacy in acute ischemic stroke, the mechanism of its dreaded limiting side effect of ICH may lay outside the vessel, in the vast and underexplored territory of the ECM (Lo et al., 2002, 2003, 2004). The NVU has been an emerging conceptual framework, which encompasses the current evidence for the mechanism of thrombolysis-related injury.

Acute ischemic stroke results in damage to the NVU. In particular, MMPs-mediated NVU injury targets the destruction of components of the vascular matrix (collagen, fibronectin, and laminin) and results in BBB breakdown, oxidative stress, and a proteolytic cascade that ultimately mediates the progression of edema and infarction (Rosenberg et al., 1992, 1996; Rosenberg, 1995, 2002; Chan, 1996, 2001; Chen and Strickland, 1997; Gasche et al., 2001; Wang and Lo, 2003) (*Figure 15-2*).

Figure 15-1

Schematic diagram of neurovascular unit (NVU). Interactions of components of the NVU Modified from WangLo, 2003

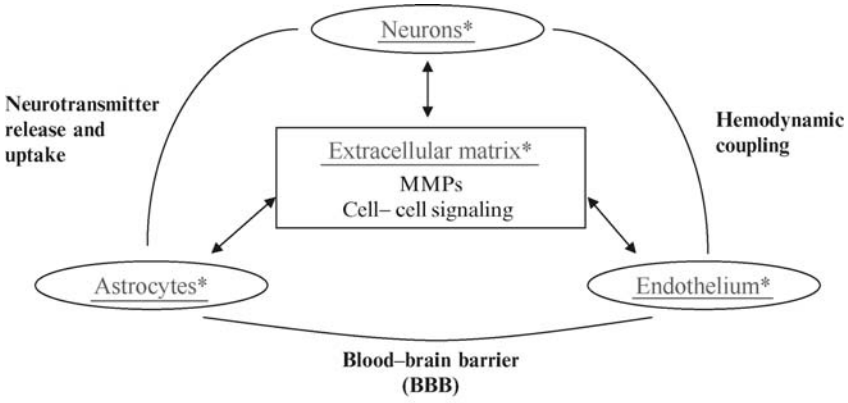
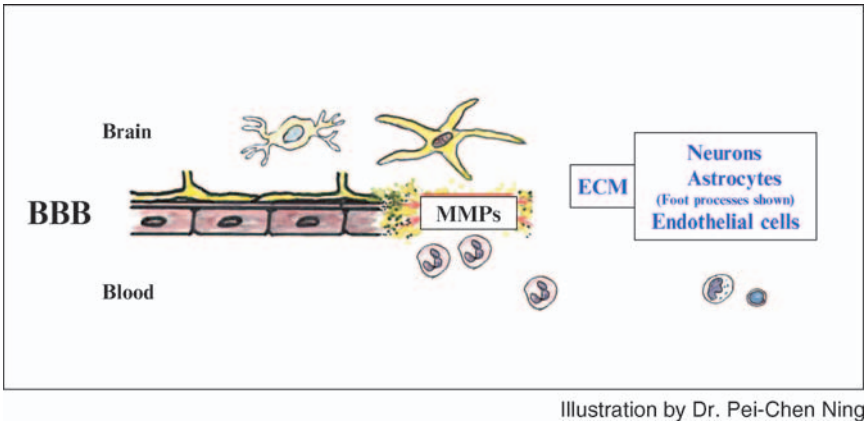


Figure 15-2

Matrix metalloproteinases (MMPs) and blood–brain barrier (BBB) in the context of the NVU. MMPs degrade components of the BBB and recruit inflammatory cells to the site of injury



Recent in vitro and in vivo data suggest that despite its proven therapeutic benefit, t-PA upregulates matrix metalloproteinase-9 (MMP-9, aka gelatinase B), an MMP implicated in NVU injury (Mori et al., 2001, 2002; Lo et al., 2002, 2003; Sumii and Lo, 2002; Montaner et al., 2003; Pfefferkorn and Rosenberg, 2003; Wang and Lo, 2003). Data in humans mirrors animal and cell-culture studies, demonstrating an association between t-PA-mediated plasma total MMP-9 upregulation and clinical outcome in thrombolyzed patients (Ning et al., 2004). One mechanism by which this pathway may impair recovery is through downstream cell–cell and cell–matrix signaling (Lo et al., 2003; Egeblad and Werb, 2002; Lee and Lo, 2004).

4.2 What are MMPs?

MMPs, characterized as zinc-dependent endopeptidases, were first discovered to degrade the tails of tadpoles as they mature into adult frogs. They comprise the largest class of proteases in humans and are even more abundant than the serine protease family of which t-PA is a member. MMPs have important functions in ECM remodeling, cell migration, and apoptosis (Nagase and Woessner, 1999; Opdenakker et al., 2001). Therefore, they have been the focus of study as therapeutic targets.

MMPs are secreted as zymogens and are under tight regulation by endogenous tissue inhibitors of MMPs called TIMPs. They degrade components of the ECM including collagen, gelatin, fibronectin, vitronectin (Yong et al., 2001). In addition, MMPs also cleave membrane-bound receptors and various cytokines (Nagase and Woessner, 1999).

MMPs are grouped roughly into five major classes and one "other" category based on their substrate specificity: collagenases, gelatinases, stromelysins, matrilysins, and membrane-type (MT) (Nagase and Woessner, 1999) (*Table 15-4*).

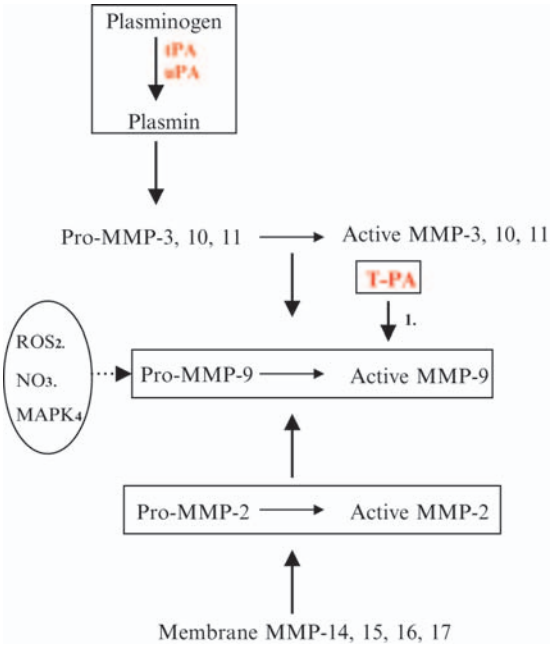
Table 15-4
Classification of matrix metalloproteinases (Nagase, 1997)

Enzyme	MMP
Collagenases	
Interstitial collagenase; collagenase 1	MMP-1
Interstitial collagenase; collagenase 2	MMP
Collagenase 2	MMP
Collagenase 4 (*Xenopus*)	MMP
Gelatinases	
Gelatinase A	MMP-2
Gelatinase B	MMP-9
Stromelysins	
Stromelysin 1	MMP
Stromelysin 2	MMP
Stromelysin 3	MMP
Matrilysins	
Matrilysin 1; Pump-1	MMP-2
Matrilysin 2	MMP-26
Membrane-type MMPs	
Transmembrane	
MT1-MMP	MMP
MT2-MMP	MMP
MT3-MMP	MMP
MT5-MMP	MMP
GPI anchor	
MT4-MMP	MMP
MT6-MMP	MMP
Others	
Macrophage elastase	MMP
No trivial name	MMP
Enamelysin	MMP
XMMP (*Xenopus*)	MMP
CA-MMP	MMP
CMMP (*Gallus*)	MMP
Epilysin	MMP

The gelatinases (MMP-2 and MMP-9) that degrade collagen IV, a major component of the basal lamina of the BBB, have been under rigorous investigation in cerebral ischemia. These gelatinases are activated by plasmin via t-PA or urokinase type plasminogen activator (uPA), linking them to the pathophysiology of t-PA-induced ICH (Murphy et al., 1992) (*Figure 15-3*).

Figure 15-3

T-PA–MMP axis: direct and indirect activation of MMP via t-PA (1) t-PA may directly activate MMP-9 via LRP mechanism (Wang et al., 2003), (2, 3) ROS, NO, MAP–Kinase are upstream regulators of MMP-9 (Morita-Fujimura et al., 2000; Gu et al., 2002; Wang et al., 2003). (4) Plasmin activates multiple MMPs, which activate MMP-9 (Lijnen, 2001)



Studies have shown that MMP is regulated upstream by several mechanisms, including oxidative stress, mitogen-activated protein (MAP) kinase pathways, and NO (Chan, 1996, 2001; Gasche et al., 2001; Gu et al., 2002; Wang et al., 2002; Maier et al., 2004).

Chan and colleagues reported that in copper/zinc superoxide dismutase (SOD1)-deficient mice, MMP proteolytic imbalance is more severe than in their WT littermates, demonstrating that oxidative stress mediates the BBB disruption through MMP activation (Chan, 2001; Gasche et al., 2001). This study links oxidative stress to MMPs and supports the finding that MMP-9 may be augmented by oxidative stress via NF-κB and AP-1 transcription sites (Wang et al., 2003). Wang and colleagues showed that MAP kinase was upregulated in rat primary cortical neurons and astrocyte cocultures and that inhibition of MAP kinase reduced the secretion of MMP-9. In addition, MMPs are also under the regulation of the NO system; *S*-nitrosylation of MMP-9's catalytic site causes direct activation. The study, by Gu and colleagues, demonstrated that nitrosative stress to the MMPs triggers anoikis-like cell death. Recently, Wang, Lo, and colleagues also demonstrated a novel low-density LRP-mediated pathway, which implicates t-PA in the direct activation of MMP-9 (Wang et al., 2004).

4.3 MMP and t-PA

The gelatinases (MMP-2, MMP-9) degrade components of the basal lamina in the BBB during cerebral ischemia, leading to edema and HT (Hamann et al., 1995, 1996 Mun-Bryce and Rosenberg, 1998; Rosenberg et al., 1998; Lo et al., 2002). In the brain, MMP-9 has been studied extensively and implicated as a major mediator of BBB disruption in animal models of focal ischemia, spontaneous ICH, and trauma (Mun-Bryce and Rosenberg, 1998; Wang et al., 2000, 2002; Asahi et al., 2001; Opdenakker et al., 2001; Aoki, 2002; Lo et al., 2002, 2003; Sumii and Lo, 2002; Pfefferkorn and Rosenberg, 2003).

In mouse models of focal ischemia, Lo and colleagues found that knockout mice deficient in MMP-9 expression were protected against BBB injury and had better poststroke motor recovery than controls (Mun-Bryce and Rosenberg, 1998; Wang et al., 2000; Asahi et al., 2001; Sumii and Lo, 2002). The MMP-9 knockout mice exhibited attenuated proteolysis of BBB and white-matter components such as myelin basic protein (MBP) isoforms (Asahi et al., 2001). Conversely, Rosenberg and others found that injection of MMP-9 increased infarct size, while broad-spectrum MMP-9 inhibition reduced both the volume of infarct and the rate of hemorrhagic conversion (Rosenberg et al., 1992, 1996, 1998; Lapchak et al., 2000).

MMP-9 has also been implicated in the pathway of t-PA-related ICH. In rat models of embolic focal ischemia, Aoki et al. offered a comparison of reperfusion methods between i.v. thrombolysis using t-PA, versus mechanical reperfusion at 2-h postocclusion (Aoki et al., 2002). Although the ischemic injury was comparable in the two groups, matrix metalloproteinase-9 (MMP-9) levels were higher in the t-PA-treated group. This suggests that t-PA may have a role in the amplification of MMP-9. In the rat MCAO model, delayed treatment (12 h) with t-PA significantly upregulated both pro-MMP-9 and active MMP-9 in comparison to earlier treatment (6 h). In particular, the combination of broad-spectrum MMP inhibitors, such as batimastat (BB-94) in conjunction with t-PA, reduced hemorrhage volumes and the incidence of t-PA-related hemorrhage, in comparison to t-PA alone (Lapchak et al., 2000; Sumii and Lo, 2002).

Mechanistically, t-PA may amplify MMP-9 via NF-κB transcription factor sites, which can be nonspecifically regulated by oxidative stress (Wang et al., 2003). Wang et al. (2003) recently demonstrated that AP-1 and NF-κB sites on MMP-9 are important for t-PA-related upregulation of MMP-9. This study showed that t-PA upregulates MMP-9 in both cell culture and WT mice. Blockade of AP-1 and NF-κB sites reduced t-PA-induced MMP-9 production. In the same experiment, RNA interference in human endothelial cell culture suggested that the MMP-9 upregulation was mediated by low-density LRP, which is abundantly expressed in the brain. These important findings offer evidence of t-PA's direct role in the upregulation of MMP-9, independent of NMDA receptor pathways. Parallel to these results, a link between endogenous t-PA and MMP-9 with HT of a brain infarction has recently been described in a photothrombotic mouse model (Zhao et al., 2004).

Furthermore, it has been reported that direct intraventricular injection of t-PA into mouse brains may bind LRP and increase BBB permeability independent of MMPs (Yepes et al., 2002). Taken together, these effects of t-PA on BBB integrity may be extremely important for stroke therapy.

In addition to BBB perturbations, endogenous t-PA-catalyzed degradation of laminin may cause an interruption in neuron–ECM interactions and predispose neurons to die via a form of apoptosis called anoikis (Frisch, 1994). In human brain endothelial cell cultures, anoikis-like death triggered by hypoxia-reoxygenation was mediated by MMP degradation of the fibronectin matrix (Lee and Lo, 2004).

Human plasma levels of total MMP-9 corroborate the in vivo animal studies. MMP-9 levels are elevated in patients with acute ischemic (Montaner et al., 2001) and hemorrhagic stroke (Abilleira et al., 2003). The total plasma levels of MMP-9 are elevated and the degree of elevation predicts cerebral ischemic HT (Abilleira et al., 2003; Castellanos et al., 2003; Montaner et al., 2003; Ning et al., 2004). In the thrombolysis patient, post-t-PA levels of total plasma MMP-9 are elevated at 8-h postsymptom onset in comparison to stroke patients not treated with t-PA (Ning et al., 2006). We have also shown that pre-t-PA treatment MMP-9 levels may predict thrombolysis-related injuries (Montaner et al., 2003) and that an increase of MMP-9 plasma levels follows t-PA treatment specially in patients with hemorrhagic complications (Montaner et al., 2004). In addition, elevated total levels of MMP-9 in stroke patients correlate both with poor thrombolysis outcome and with later elevation of inflammatory markers (Ning et al., 2004). MMPs may be a promising marker of stroke outcome and have the potential to help triage thrombolysis therapy.

5 Conclusions

Stroke is a major source of long-term disability and is one of the leading causes of mortality in the USA. In an aging population, the incidence of stroke doubles every decade after 55 years of age (Association, 2001). However, t-PA, the only FDA-approved treatment for acute ischemic stroke, carries a grave consequence of ICH in certain patients. The clinically imposed therapeutic window of 3 h lacks specificity in patient selection and bars the majority of acute stroke patients from the benefit of t-PA. Especially with the encouraging data from the recent meta-analysis, which confirms the benefit of thrombolysis up to 4.5-h poststroke, it is of utmost importance to find ways to target a broader population (Hacke et al., 2004).

On the basis of animal, cell-culture, and human data, MMPs play an active role in t-PA-related injuries. Emerging data now suggest that some of these potentially neurotoxic side effects of t-PA may be due to its signaling actions in the NVU (Lo et al., 2003, 2004; Wang et al., 2004). Besides its intended role in clot lysis, t-PA is also an extracellular protease and signaling molecule in the brain. t-PA mediates matrix remodeling during brain development and plasticity. By interacting with the NMDA-type glutamate receptor, t-PA may amplify potentially excitotoxic calcium currents.

In the context of the NVU, t-PA may degrade ECM integrity and increase risks of neurovascular cell death, BBB leakage, edema, and hemorrhage. Understanding these pleiotropic actions of t-PA may reveal new therapeutic opportunities for combination stroke therapy.

Animal models of focal ischemia demonstrate that cotreatment with t-PA plus MMP inhibitors ameliorated reperfusion injury. Alternatively, blocking neurotoxic properties of t-PA with neuroserpin, a neuronal serine protease inhibitor, increased the time-to-treatment window for thrombolysis in rat stroke models. Other thrombolytic agents, such as microplasmin or vampire bat salivary plasminogen activator, may not trigger MMP dysregulation or enhance excitotoxic neurodegeneration. These approaches may optimize t-PA thrombolysis by reducing the risks of hemorrhage, widening the treatment window, offering new predictors for patient selection, and providing venues for the discovery of new therapeutic targets.

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