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Endogenous Agents That Contribute to Generate or Prevent Ischemic Damage

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1. Introduction

From single to multicellular organisms, protective mechanisms have evolved against endogenous and exogenous noxious stimuli. Over the past decades numerous signaling pathways by which the brain senses and reacts to such insults as neurotoxins, substrate deprivation and inflammation have been discovered. Research on preconditioning is aimed at understanding endogenous neuroprotection to boost it or to supplement its effectors therapeutically once damage to the brain has occurred, such as after stroke or brain trauma. Another goal of establishing preconditioning protocols is to induce endogenous neuroprotection in anticipation of incipient brain damage. Currently several endogenous neuroprotectants are being investigated in controlled clinical trials. There is consensus that many of the neuroprotectants, which were highly effective in animal models of stroke, but failed in clinical trials, were unsuccessful because of side effects, which in many cases led to premature termination of the trial. Nowadays research aims to overcome this problem by developing compounds which induce, mimic, or boost endogenous protective responses and thus do not interfere with physiological neurotransmission. In the present review we will give a short overview on the signals, sensors, transducers, and effectors of endogenous neuroprotection. We will first focus on common mechanisms, on which pathways of endogenous neuroprotection converge. We will then discuss various applications of endogenous neuroprotectors and explore the prospects of endogenous neuroprotective therapeutic approaches.

2. Phisiopatology of cerebral ischemia

Development of stroke prophylaxis involves the understanding of the mechanisms of damage following cerebral ischemia and elucidation of the endogenous mechanisms that combat further brain injury (FIGURE 1).
The binding of glutamate to its receptors and the activation of voltage-gated $Ca2^{+}$ channels (VGCC) causes calcium to influx into the cell. Calcium is among the mediators that initiate the genomic response to cerebral ischemia. The superoxide dismutase (SOD) gene is upregulated to neutralize the reactive oxygen species (ROS). The generation of nitrous oxide (NO) in the neuron is cytotoxic. The interaction between antiapoptotic genes, such as Bcl-2, and proapoptotic genes, such as Bax, determines whether cytochrome c will be translocated

from the mitochondria to the cytosol. In the cytosol, cytochrome c combines with Apaf-1 to activate the caspases. Proinflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-a (TNF-a), are generated. Survival pathways involving growth factors (GFs), immediate early genes (IEGs), and heat shock proteins (Hsps) are also stimulated. Ultimately, the activation of these genetic pathways determines the fate of the ischemic cell.

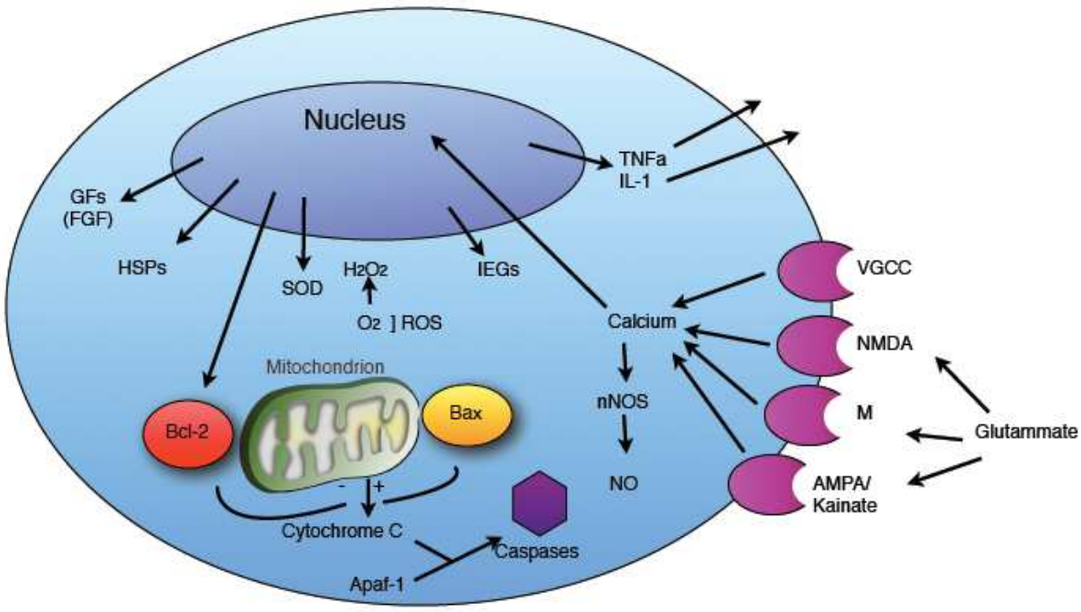

Fig. 1. Phisiopatology of cerebral ischemia

2.1 Excitotoxicity and calcium

Although the brain represents only 2% of body weight, it uses an astonishing 20% of the body's oxygen in adults. The innumerable cells of the brain require an almost continuous flow of oxygen and glucose, making them exquisitely sensitive to any interruption in energy supply. Energy depletion and reduced levels of adenosine triphosphate initiate a series of events that cause cells to die. Glutamate, the main excitatory neurotransmitter of the central nervous system, is a trigger of neuronal loss during stroke. During ischemia, an excess of glutamate is released into the extracellular space. The mechanism to clear glutamate is energy dependent; glutamate quickly builds to toxic levels when energy is depleted. Glutamate causes ionic shifts; Na^+ enters the cell and K^+ exits. Water passively follows the influx of Na^+ leading to cellular swelling and edema. The membrane potential is lost and the cell depolarizes. In the ischemic core, cells undergo anoxic depolarization and never repolarize. However, cells in the penumbra initially retain the ability to repolarize so that they may depolarize again. As cells in the penumbra undergo these peri-infarct depolarizations the energy supply and ionic homeostasis are further compromised, resulting in an increase in the size of the ischemic lesion. Glutamate activates three main families of receptors: N-methyl-d-aspartate (NMDA), a-amino-3-hydroxy-5 methylisoxazole/kainate, and metabotropic glutamate receptors. Activation of these receptors leads to a buildup of Ca^{2+} within the cell. Ischemia therefore triggers glutamate receptor-mediated excitotoxicity and Ca^{2+} overload within the cell. Originally, neuronal death from excitotoxicity was

believed to result from depletion of cellular energy stores from overexcited neurons. However, the influx of Ca^{2+} seems to be the major pathogenic event contributing to cell death. This translocation of Ca^{2+} is accomplished through glutamate, particularly through the NMDA receptor, as well as through voltage-gated Ca^{2+} channels that open after cell depolarization. Calcium channel antagonists have displayed neural protection in animal models but have not shown benefit in clinical trials partially because they were administered too late after stroke onset or in insufficient quantity. However, it appears that Ca^{2+} influx into the cell is only the initial step in a complex biochemical cascade.

2.2 Free radicals

Reactive oxygen species are produced after the induction of ischemia and upon reperfusion. The oxidative stress produced by the reactive oxygen species destroys the cell through lipid peroxidation, protein oxidation, and DNA damage. Certain endogenous antioxidants scavenge and neutralize the reactive oxygen species. In particular, the antioxidant superoxide dismutase detoxifies the superoxide ($O2^{-}$) free radical by converting it to hydrogen peroxide (H2O2). Glutathione peroxidase can then convert H2O2 into oxygen and water. During times of oxidative stress, the superoxide dismutase gene is upregulated. Neural protection strategies have included both the administration of exogenous superoxide dismutase and manipulation of the superoxide dismutase gene family. Nitric oxide (NO) is another free radical that is increased during ischemia due to an increase in intracellular Ca^{2+}. The formation of NO is catalyzed by the enzyme NO synthase (NOS). NOS has several isoforms—a neuronal type (nNOS) located in neurons and an endothelial type (eNOS) in the vascular endothelium. NO is also generated in microglia, astrocytes and invading macrophages after the induction of an inducible isoform (iNOS). Initially after ischemia, the formation of NO in the vascular endothelium by eNOS may improve CBF through vasodilatation offering neuroprotection. However, synthesis of NO by nNOS and iNOS is cytotoxic, leading to an inhibition of mitochondrial respiration, glycolysis, and DNA synthesis. Because of the dual role of NO in cerebral ischemia, neuronal protection strategies need to target the specific isoform of NOS. For instance, deletion of the nNOS or iNOS gene in animal models has provided neuronal protection.

2.3 Apoptosis

After an ischemic event, cells in the penumbra may initiate a program of autodestruction known as apoptosis. Apoptosis occurs in the developing brain. More than half of progenitor neurons undergo this process of programmed cell death while forming neural circuits. During ischemia, cells in the ischemic core undergo necrosis while cells in the ischemic penumbra may actually self destruct through this process of apoptosis. The mitochondria is regarded as the apoptotic headquarters of the cell. One of the key events in apoptosis is the translocation of cytochrome c from the intermembrane of the mitochondria into the cytosol. In the cytosol, cytochrome c combines then with apoptotic activating factor (Apaf-1) to activate a set of proteases known as caspases. These caspases actually dismantle the cell during apoptosis. A family of death-promoting genes, known as the Bcl-2 family, determines whether a cell will undergo apoptosis. The Bcl-2 gene is antiapoptotic and prevents the translocation of cytochrome c and activation of caspases. However, the Bax gene (one of the members of the Bcl-2 family) is proapoptotic, facilitating the translocation of cytochrome c and apoptosis. During ischemia, proapoptotic genes such as Bax are

activated, resulting in the autodestruction of the cell. Thus, neuronal protection may be gained through blocking these death-promoting genes. Other strategies include giving caspase antagonists or preventing the translocation of cytochrome c from the mitochondria. Preventing apoptosis in the penumbra is another effective technique in animal models for neuronal protection.

2.4 Inflammation

The inflammatory response may be an important part of the ischemic cascade. Soon after the onset of stroke, leukocytes invade the ischemic zone. The mechanisms by which these inflammatory cells contribute to the evolution of ischemia include microvascular occlusion by adherence to the endothelium, producing cytotoxic enzymes and generating injurious free radicals. Cytokines are intracellular messengers that mediate the recruitment of the leukocytes and the induction of adhesion molecules. The two main proinflammatory cytokines are interleukin-1 (IL-1) and tumor necrosis factor-a (TNF-a). The adhesion molecules that facilitate the movement of leukocytes along the surface of the endothelium are the E and P selectins, whereas intracellular adhesion molecules attach the leukocytes to the endothelium so that they may leave the vascular space and enter the site of injury. Research has been focusing on the manipulation of these proinflammatory cytokines and adhesion molecules to provide neuronal protection.

2.4.1 Survival pathways

The cytokines that are activated during ischemia also include growth factors that actually promote neuronal survival and, in some cases, neuronal outgrowth and synapse formation. Fibroblast growth factor is the most extensively studied growth factor. Although the exact mechanism of neuroprotection of fibroblast growth factor is not fully understood, it includes upregulation of free radical scavenging enzymes and Ca^{2+} binding proteins, downregulation of the NMDA receptor and vasodilatation. The administration of growth factors has provided cerebral protection in animal models. Because they exert both protective and trophic influences on neurons, growth factors remain an exciting prospect in drug development for stroke. Other gene families and proteins are activated during ischemia. Immediate early genes, such as those of the Fos and Jun families, are activated soon after ischemia. It is believed that Ca^{2+} and reactive oxygen species are involved in the expression of immediate early genes. Although the exact role of each of the immediate early genes in ischemia is not yet understood, they are known to participate in apoptosis. Some immediate early genes may even afford neuronal protection. Ischemia also induces the expression of molecular chaperones known as heat shock proteins, which maintain protein function and assist in protein transport in response to injury. Increasing the expression of heat shock proteins to combat ischemia has been attempted.

3. Erythropoietin

The hormone erythropoietin (Epo) is a 165-amino acid (~30 kDa) glycoprotein that belongs to the cytokine type I superfamily. Originally, it was believed that the only role of Epo was the regulation of erythropoiesis. This role is attributed to the ability of Epo to inhibit programmed cell death (apoptosis) in erythroid cells and thus allow the maturation of erythrocytes. Since blood oxygen availability is the main regulator of erythropoiesis,

hypoxia induces the gene expression of Epo in the kidney, the main site for Epo production, and in the liver (Cotena et al, 2008) in a negative feedback system between the kidney and the bone marrow. Research performed in the last decade has shown that Epo and its receptor (EpoR) are expressed in tissues other than those involved in erythropoiesis. These include the brain, the reproductive tract (Kobayashi et al, 2002; Marti et al, 1996; Masuda et al, 2000), the lung, the spleen, and the heart (Fandrey and Bunn, 1993). Accordingly, a novel cytoprotective effect of Epo was established in several organs. For example, Epo reduced injury and dysfunction after ischemia-reperfusion in the mouse kidney (Patel et al, 2004), and it showed protection in various myocardial ischemia models (Bogoyevith,2004; Cai et al, 2003; Parsa et al, 2003).

3.1 Epo/EpoR expression and regulation

Epo is mainly produced in the interstitial fibroblasts in the adult kidney and the hepatocytes of the fetus, whereas EpoR is normally expressed in erythroid precursor cells in the bone marrow (Marti, 2004). However, recent data have shown that the expression of Epo and its receptor, EpoR (both mRNA and protein), coincides in the same organ and even within the same cell. Epo and EpoR expression are widely distributed in the mammalian brain (Genc *et al*, 2004; Marti, 2004), albeit at lower levels than in the kidney (Brines and Cerami, 2005). Epo thus has to be added to the growing list of hematopoietic growth factors found to be expressed and act in the central nervous system (CNS).

3.2 Expression of Epo/EpoR in the brain

Epo/EpoR mRNA and protein were detected is several regions of the murine and primate brain, including cortex, hippocampus and amygdale, cerebellum, hypothalamus, and caudate nucleus (Siren et al, 2001). With respect to the type of cells in the brain that express Epo, astrocytes are the main source of Epo in the brain (Masuda et al, 1994). Moreover, it has been shown in vitro and in vivo that neurons express Epo (Bernaudin et al, 1999, 2000). Similarly, EpoR is expressed on neurons and astrocytes. In addition, primary cultures of human neurons, astrocytes, and microglia express EpoR mRNA (Nagai et al, 2001), and EpoR expression was also detected in primary cultures of rat oligodendrocytes (Genc et al, 2006). In addition to neurons, oligodendrocytes, and glial cells, a strong immunoreactivity for EpoR was found to be associated with brain vascular endothelial cells, showing that these cells also express EpoR (Brines et al, 2000). These findings implicate a broad spectrum of actions of Epo in the brain.

3.3 Regulation of Epo/EpoR expression

As mentioned above, Epo is upregulated in response to hypoxia. As, for many of the hypoxic adaptation processes in the body, the regulation of Epo expression is based on the transcriptional regulation of two hypoxia-inducible factors HIF-1 and HIF-2 (Wenger, 2000). HIFs are heterodimers composed of an α- and a β -subunit. Two forms of the oxygen-labile α exist, 1α and 2α. The α-subunit is stabilized under hypoxic conditions leading to the binding of the heterodimer HIF-1 or HIF-2 to specific DNA sequences located in the hypoxia response elements of target genes such as Epo or vascular endothelial growth factor (VEGF) (Wenger, 2002). Although HIF-1α was originally identified as the transcription factor responsible for Epo expression (Semenza et al, 1991), more recent evidence suggests that Epo is a target of HIF-2 (Eckardt and Kurtz, 2005). The stability of HIF-α is regulated by

enzymatic hydroxylation of specific amino acids on the α subunit by a group of oxygenases (FIGURE 2). Under normoxic conditions, a specific prolyl hydroxylation within the oxygen-dependent degradation domain of HIF-α takes place. This prolyl hydroxylation allows binding of the von Hippel-Lindau protein (pVHL), leading to ubiquitylation and proteasomal degradation of the HIF-α subunit (Ivan et al, 2001; Jaakkola et al, 2001). The enzymes responsible for this hydroxylation are termed prolyl hydroxylase domain enzymes (PHD1-3) (Bruick and McKnight, 2001; Epstein et al, 2001) and are widely expressed. Furthermore, in the presence of oxygen, another hydroxylation reaction takes place on an asparaginyl group in the COOH-terminal transactivation domain of HIF-α, blocking its binding to the transcriptional coactivators (Lando et al, 2002a). This process is governed by a specific asparaginyl hydroxylase termed factor-inhibiting HIF (FIH) (Hewitson et al, 2002; Lando et al, 2002b). So, under normoxia, FIH and PHD(s) are active, leading to transcriptional inactivation and degradation of HIF-α, whereas under hypoxic conditions both enzymes are inactive. HIF is then stabilized and able to induce the expression of target genes, including Epo.

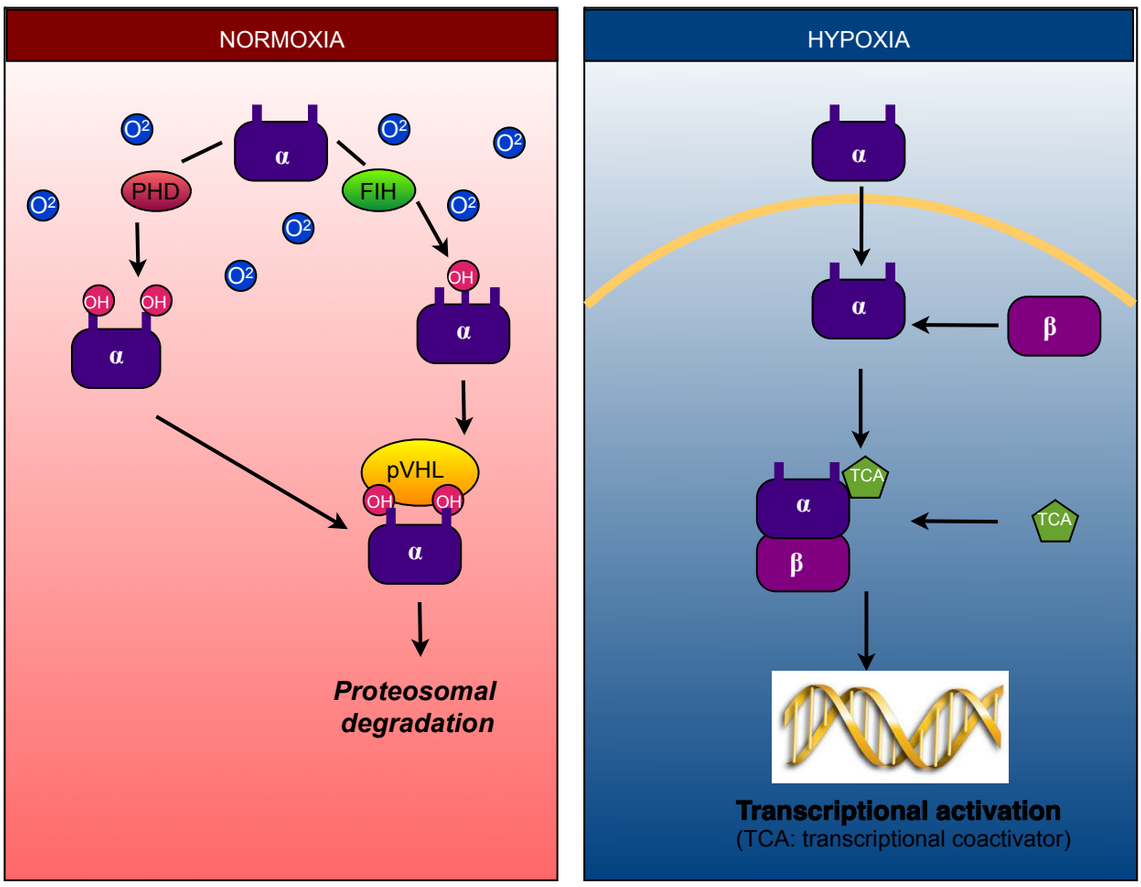

Fig. 2. Under normoxic conditions, specific prolyl hydroxylation within the oxygen-dependent degradation domain of HIF- α takes place. By contrast, under hypoxic conditions, FIH and PHD(s) are both inactive, and HIF is stabilized and able to induce the expression of target genes including Epo.

This basic mechanism of regulation seems to be of relevance for brain-expressed Epo, since in several experimental systems Epo was upregulated under hypoxic conditions in the brain of several mammalian species including mouse, rat, monkey, and human (Marti et al, 1996, 2000; Siren et al, 2001). However, depending on the severity of hypoxia, Epo mRNA level can increase 3- to 20-fold in the brain in contrast to 200-fold in the kidney. Moreover, although the increase in Epo expression in the kidney seems to be transient with a decrease after 8 h of continuous hypoxia, the level of Epo in the brain remains high for at least 24 h (Chikuma et al, 2000). This indicates a tissue-specific degree of regulation. Indeed, although HIF-1α levels in the kidney under systemic hypoxia peak after 1 h and again reach basal levels 4 h thereafter, in the brain the HIF-1α peak level is reached after only 5 h and returns to the basal level not before 12 h (Stroka et al, 2001). A possible explanation for the different time course in the brain might be an altered composition of the various PHD forms. It has to be noted that hypoxia is not the only factor activating HIF. Several studies have shown that pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) or lipopolysaccharide (LPS) induce the expression of HIF (Frede et al, 2007). With regard to the EpoR, it is regulated by pro-inflammatory cytokines (Nagai et al, 2001), such as TNF-α, IL-1β, and Epo itself (Chin et al, 2000). The role of hypoxia in the regulation of EpoR expression is controversial. Whereas we did not observe hypoxic induction of EpoR expression in neurons or astrocytes (Bernaudin et al, 2000), anemic stress induced EpoR expression in the brain of human EpoR transgenic mice (Chin et al, 2000). Moreover, in the same study, hypoxia increased EpoR expression in neuronal cells in vitro. The mechanism of hypoxic EpoR regulation remains to be established, since EpoR has not been identified as HIF target gene so far.

3.4 Epo signaling

Epo promotes cell survival through inhibiting apoptosis (FIGURE 3).

In erythroid cells, after binding of Epo to its receptor (EpoR), Janus tyrosine kinase 2 (JAK2) is phosphorylated and thus activated. This leads to engaging secondary signaling molecules such as signal transducer and activator of transcription 5 (STAT5), followed by the activation of Ras mitogen-activated protein kinase (MAPK), ERK-1/-2, and PI3K/Akt (22). Moreover, Epo induced the upregulation of the anti-apoptotic protein BCL-XL (Kilic et al, 2005). The functional significance of these signaling molecules in erythropoiesis is not absolutely clear though. For instance, whereas in one study STAT5 knockout adult mice were largely unaffected in their erythroid lineage (Teglund et al, 1998), in another study STAT5 knockout embryos suffered from severe anemia, showed a reduced number of erythroid progenitors cells, and had higher numbers of apoptotic cells (Socolovsky et al, 1999). Most of these pathways seem also to be functional in the brain (Brines and Cerami, 2005; Kilic et al, 2005). In vitro, inhibition of MAPK and PI3K blocked Epo-mediated protection of rat hippocampal neurons against hypoxia (Siren et al, 2001b). Moreover, using ERK-1/-2 and Akt inhibitors, Kilic et al. showed that activation of these proteins is essential for Epo-mediated neuroprotection in an animal model of focal cerebral ischemia. The role of STAT5 in Epo-induced neuroprotection is, however, controversial. STAT5 phosphorylation has been shown to occur in hippocampal CA1 neurons after transient global cerebral ischemia in rats (Zhang et al, 2007). Therefore, the authors concluded that STAT5 plays a role in Epo-mediated neuroprotection. However, in a very recent study, in an in vitro model of glutamate toxicity using hippocampal neuronal culture from STAT5 knockout mouse

fetuses, STAT5 was not required for Epo-mediated neuroprotection (Byts et al, 2008). However, STAT5 was indispensable for the neurotrophic function of Epo. A unique pathway for the brain seems to be that activation of EpoR induces nuclear factor- κB (NF-κB) translocation into the nucleus and that this effect is important for Epo-mediated neuroprotection (Digicaylioglu and Lipton, 2001). Interestingly, Epo-induced NF-κB translocation was observed only in neuronal cells and not in astrocytes. Thus it appears likely that NF-κB, in the nucleus, induces the expression of neuroprotective and anti-apoptotic proteins. However, some differences exist between the signaling cascade activated by Epo in the CNS and in erythroid cells. For instance, in one study, BCL-XL has been found to be important in Epo-mediated protection of erythroid but not neuronal cells (Rischer et al, 2002). Additionally, Epo has been found to activate phospholipase C-gamma (PLCγ) (Marreo et al, 1998) and thus can directly influence neuronal activity (Koshimura et al, 1999) and neurotransmitter release (Kawakami et al, 2000) by modulating intracellular calcium concentrations in neurons.

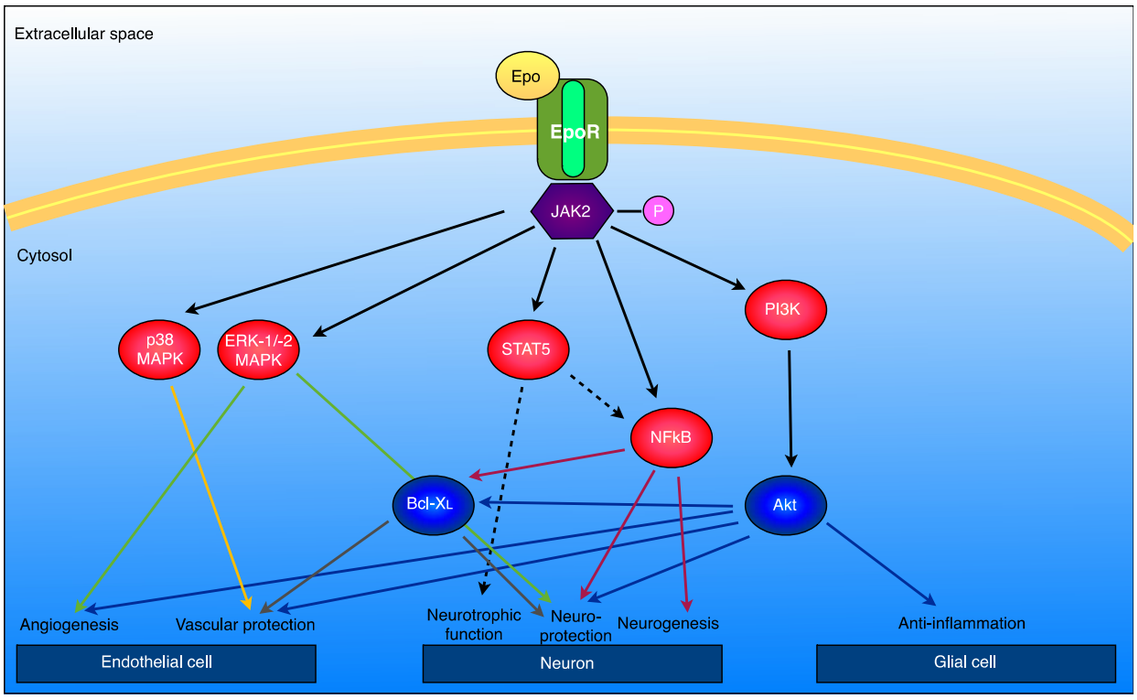

Fig. 3. Epo Signaling

3.5 Epo function in the CNS

For almost a century, Epo was thought to be involved in the process of erythropoiesis only. Through its antiapoptotic action, it enables committed erythroid progenitor cells to survive and mature (Jelkmann, 1992). However, during the last decade, it became evident that Epo is implicated in other processes such as neuroprotection, neurogenesis, and angiogenesis, and plays an important role as neurotrophic as well as immunomodulatory factor. The important role of Epo in the CNS is also evident from studies with EpoR knockout mice. As a result of EpoR deficiency, these mice show massive apoptosis and a reduction in the

number of neuronal progenitor cells (Yu et al, 2002). A comprehensive description of the role of the Epo/EpoR system in development is found elsewhere (Arcasoy, 2008; Dame et al, 2001).

3.5.1 Neuroprotection

For a long time, Epo has been used clinically in patients suffering from anemia due to end-stage renal failure. In addition to the correction of anemia, these patients showed improved cognitive abilities (Siren and Ehrenreich, 2001). Initially, since it was believed that systemic Epo cannot pass through the blood-brain-barrier due to its large size (Recny et al, 1987) and brain-derived Epo production and expression of EpoR in the CNS were not yet discovered, the positive effect on cognition was attributed to the improved oxygen-carrying capacity of the blood after Epo-induced erythropoiesis. However, since later studies have shown that both Epo and its receptor are expressed in different regions of the brain by different cell types (Genc et al, 2004; Marti, 2004), the hypothesis was established that locally produced as well as exogenously added Epo could directly influence cognitive function. Interestingly, the expression level of Epo and EpoR is especially high in regions of the brain known to be particularly sensitive to acute hypoxia (Lipton, 1999), the hippocampus and the telencephalon (Digicaylioglu et al, 1995), suggesting that Epo might act as a protective agent against hypoxia. Indeed, infusion of soluble EpoR (capable of binding with endogenous EPO, thus sequestering it) into the brain of gerbils, which were subjected to a mild form of ischemia that normally does not cause neuronal damage, resulted in neuronal death in the hippocampus, clearly showing that endogenous Epo has a neuroprotective effect (Sakanaka et al, 1998).

3.5.2 Neurotrophic function and neurogenesis

Besides neuroprotection under hypoxic conditions, Epo also has a neurotrophic function in normoxic neurons. This was first demonstrated by Konishi and co-workers showing that Epo augments the activity of choline acetyltransferase in primary cultured mouse septal neurons (Konishi et al, 1993). Epo promoted the regeneration of septal cholinergic neurons in adult rats that had undergone fimbria-fornix transections. In addition and similar to its anti-apoptotic role in erythropoiesis, Epo promoted the survival and differentiation of dopaminergic precursor neurons in vitro (Studer et al, 2000). Moreover, hypoxia-induced Epo production appeared to directly act on neuronal stem cells in the forebrain, showing that Epo plays a direct role in neurogenesis after hypoxia (Shingo et al, 2001). In addition, Epo also acts indirectly by inducing brain-derived neurotrophic factor (BDNF) expression (Wang et al, 2004), which in turn augmented the effect of Epo on neurogenesis. These data show that Epo is not only involved in neuroprotection, but also in neuronal survival, differentiation, and neurogenesis.

3.5.3 Angiogenesis and vascular permeability

Besides its direct effects on neurons, Epo-induced neuroprotection may be attributed to an improvement in brain perfusion by promoting new vessel growth. Anagnostou et al demonstrated mitogenic and chemotactic effects of Epo on human umbilical vein and bovine adrenal capillary endothelial cells (Anagnostou et al, 1990). Moreover, Epo stimulated vessel outgrowth of rat aortic rings (Carlini et al, 1995), suggesting that Epo has angiogenic effects. This was further supported by the observation that Epo injection into the

mouse uterine cavity stimulated neovascularization in the endometrium (Yasuda et al, 1998). Similarly, neovascularization was stimulated in the chick embryo chorioallantonic membrane upon Epo administration (Ribatti et al, 1999a). The angiogenic effect of Epo was also found in the brain, since capillary endothelial cells express two forms of EpoR mRNA and Epo showed a dose-dependent mitogenic activity on brain capillary endothelial cells (Yamaji et al, 1996). This angiogenic effect was finally confirmed in mice genetically engineered to lack either Epo or its receptor (EpoR) where mutant embryos suffer from severe defects in angiogenesis (Kertesz et al, 2004). In addition to its angiogenic effect, Epo is involved in the regulation of vascular permeability. In an in vitro model of the blood-brain barrier (BBB), Epo treatment protected bovine brain endothelial cells against VEGF-induced increase in vascular permeability (Martinez-Estrada et al, 2003). This suggests that the protective effect of Epo on the brain could be mediated by stimulating angiogenesis as well as by protecting the BBB.

3.5.4 Anti-inflammation

Inflammatory processes play a major role in the pathogenesis of cerebral ischemia, where Epo is protective. Inflammation results in influx of leukocytes from the blood into the brain and in activation of resident microglial cells (Dirnagl et al, 1999). These cells produce inflammatory mediators and cytokines leading to barrier damage, microvascular occlusion, and thus the aggravation of the injury (Witko-Sarsat et al, 2000). In an animal model of cerebral ischemia, administration of Epo resulted in the reduction of the local production of TNF, IL-6, and the chemokine MCP-1, all markers of inflammation, subsequently leading to a marked reduction of infarct size. These results indicate that Epo has an anti-inflammatory effect that contributes to its direct neuroprotective effect during cerebral ischemia (Villa et al, 2003). Since Epo did not reduce cytokine production in response to LPS applied directly in vivo and in vitro, the authors concluded that the observed antiinflammatory effect is due to inhibiting neuronal apoptosis and not to a direct effect on inflammatory cells. Epo might reduce leukocyte transmigration through endothelial cells, since Epo enhances the resistance of endothelial cells toward ischemia (Chong et al, 2002). The protective effect of Epo on oligodendrocytes against cytotoxicity induced by inflammatory stimuli (Genc et al, 2006) could explain the beneficial effect of Epo in case of MS where oligodendrocytes play a crucial role in the pathogenesis of the disease.

3.5.5 Transport through BBB

An important prerequisite for considering Epo as a therapeutic agent in CNS diseases is to answer the question as of whether Epo, administered systemically, is able to cross the BBB. Brines et al. (Brines et al, 2000) injected mice with biotinylated Epo and subsequently visualized brain section with peroxidase-labeled streptavidin. Indeed, a signal for biotin was detected in a region surrounding the capillaries extending into brain parenchyma. The authors concluded that Epo crosses the BBB. However, biotin might not be an ideal tool to study BBB permeability since it is rapidly transported across the BBB (Shi et al, 1993; Spector and Mock, 1987), and, therefore, even a small amount of free biotin in the blood will cross the BBB leading to false results. Since the authors detected EpoR in the brain capillaries, they attributed Epo transport through BBB to transcytosis. This hypothesis was later challenged by the observation that radiolabeled Epo and albumin crossed the BBB and entered the brain parenchyma in similar kinetics, showing that the transport of Epo across BBB is rather

mediated by the extracellular pathways (Banks et al, 2004). However, variations in physiological serum Epo level may not result in significant changes of Epo levels within the brain, since no correlation between serum and liquor Epo concentrations was found when the BBB is intact (Marti et al, 1997). In summary, one can conclude that endogenously produced Epo (by kidney or liver) has only a marginal influence on brain Epo availability, whereas high dosages of therapeutically administered r-hu Epo can penetrate even the intact BBB (Marti et al, 1997). Accordingly, many studies are currently ongoing to test the therapeutic potential of Epo in many CNS diseases.

3.6 Epo in stroke

The first hint came from the observation that the expression of Epo and its receptor in the brain is upregulated upon cerebral ischemia (Bernaudin et al, 1999; Siren et al, 2001). Several in vivo experiments confirmed this hypothesis. Intracerebroventricular injection of Epo 24 h before permanent occlusion of the MCA in mice reduced infarct volume significantly. Similarly, infusion of Epo in the lateral ventricles of gerbils in a global ischemia model rescued hippocampal CA1 neurons and increased the number of synapses in the same region (Sakanaka et al, 1998). Moreover, in another experimental rodent model of cerebral ischemia where the MCA is transiently occluded, systemic administration of Epo also reduced the infarct size (Brines et al, 2000). Significantly, this protective effect of Epo was retained even when Epo was applied 6 h after the onset of the cerebral ischemia. In addition, brain-specific overexpression of Epo reduced infarct size in mice subjected to transient cerebral ischemia (Kilic et al, 2005). Other studies, where the functional outcome of Epo treatment was investigated, have shown that Epo not only reduces infarct volume but also improves the learning ability in gerbils and reduces the navigation disability in rats (Sadamoto et al, 1998; Sakanaka et al, 1998). Epo has also been shown to be protective in models of hemorrhagic stroke where the interruption of the cerebral blood flow is due to subarachnoid or cerebral hemorrhage (Alafaci et al, 2000; Grasso et al, 2002). The above-mentioned studies prompted the initiation of clinical trials in stroke patients. The safety and proof-of-concept phases of the Göttingen-Epo-Stroke Study have shown Epo to be safe and to improve the patient functional outcome after stroke (Ehrenreich et al, 2002). Although good evidence for direct neuroprotection exists, the observed brain-protective effect of Epo could also be attributed to its effect on astrocytes. Astrocytes protect neurons from oxidative stress by neutralizing reactive oxygen species (Dringen and Hirrlinger, 2003). It has been reported that activated astrocytes in ischemic human brain express increased levels of EpoR (Siren et al, 2001). Since Epo enhances brain glutathione peroxidase activity (Kumral et al, 2005), Epo, by binding to EpoR on the surface of activated astrocytes, might contribute to the astrocyte-mediated neuroprotective effect against ischemia-induced free-radical formation.

3.7 Safety concerns with the clinical use of EPO

Clinical studies are ongoing to test the safety and efficacy of EPO for the treatment of different neurological diseases. In the recent multicenter Epo stroke trial (Ehreich et al, 2009), adult stroke patients receiving Epo after tissue-plasminogen activator (t-PA)-induced thrombolysis reported increased mortality, intra-cerebral hemorrhage, brain edema, and thromboembolic events. The increased death rate in the rtPA population is still unexplained and may result from a combinant of factors and/or potential rtPA-EPO interactions. In contrast, in non rtPA population, the tendency toward a higher death rate in the EPO group

might be explained by higher stroke severity of dead patients on inclusion (before any study medication was applied). Moreover, the mechanism of action of EPO is different from the clot-dissolving strategy pursued by thrombolysis. It would, therefore, have been most attractive to see that the neuroprotective approach using EPO, aimed at salvaging potentially viable brain tissue from spreading of death signals, and thrombolysis, targeting reopening of the feeding artery, had provided additive beneficial outcome. However, the unexpected observation that a combination of EPO and rtPA is not advantageous, and can even be detrimental, poses at present a contraindication for acute EPO treatment in patients receiving rtPA.

4. Albumin

Albumin is the most abundant plasma protein synthesized mainly in the liver. Albumin is also a major component of most extracellular fluids including cerebrospinal fluid (CSF), interstitial fluid (ISF) and lymph. It is a non-glycosylated and negatively charged protein with high ligand binding and transport capacity. It has multifunctional properties which include the maintenance of colloid osmotic pressure of plasma, transportation of hormones, fatty acids, drugs and metabolites, regulation of microvascular permeability, antioxidant activity, anti-thrombotic activity and anti-inflammatory activity (Evans, 2002; Garcovich et al, 2009). Owing to its multifunctional properties it has been widely used in therapeutics related to hepatology. The volumeexpanding property of albumin, in combination with other therapeutic approaches, has been used for the clinical benefit of patients with liver cirrhosis. Also, human serum albumin (HSA) as an iso-oncotic (4-5%) solution has been used to combat blood volume deficits and as a hyperoncotic (20-25%) solution has been used for restoration of oncotic deficits (Arroyo, 2002; Garcovich et al, 2009). Albumin has been shown to play a crucial role in the microcirculation of many organs including brain. Owing to its strong hemodynamic and binding capacity, it has been implicated in physiological and many disease conditions of the brain. Albumin has been implicated in neurological diseases such as ischemic stroke, Alzheimer's disease and epilepsy. High-dose human albumin is robustly neuroprotective in preclinical ischemia models and it is currently in Phase III clinical trials for acute ischemic stroke (Ginsberg, 2008). Albumin also has the potential to produce direct neuroprotective action on neuronal and glial cells.

4.1 Albumin synthesis and distribution

Albumin protein contains a single polypeptide chain of 585 amino acids with a molecular mass of approximately 67 kDa.

In the mouse liver, the albumin gene becomes active during early foetal stages and the transcript levels gradually increase after birth until high levels are reached in the adult animal (Tilghman and Belayew, 1982). Albumin is synthesized as preproalbumin in the liver, which has an N-terminal peptide that is removed before the nascent protein is released from the rough endoplasmic reticulum. The product, proalbumin, is in turn cleaved in the Golgi vesicles to produce albumin. Albumin synthesis predominantly occurs in the liver at the rate of 10-15 g/day. In healthy human adults, total albumin content is approximately 250-300 g/70 kg of the body weight and the majority of synthesized albumin (40-45 %) is maintained in the plasma. A very small amount of albumin is stored in liver (< 2 g) and the remaining amount is located in the muscle and skin (Quinlan et al, 2005). Albumin synthesis

is regulated at both the transcriptional and post-transcriptional levels and this regulation is important to meet the demands of plasma, because albumin is not stored in the liver in large amounts. The rate of albumin transcription is affected by several conditions such as trauma, sepsis, hepatic diseases, diabetes and fasting. The change in interstitial colloid oncotic pressure is thought to be the predominant factor for regulation of albumin synthesis. Albumin homeostasis is maintained by balanced catabolism occurring in all tissues but most of the albumin (40-60 %) is degraded in the muscle and skin. However, the liver (15 %), kidney (10 %) and gastro-intestinal tract (10 %) are also responsible for albumin degradation. Albumin leaks from plasma at a rate of 5 % per hour and is returned to the vascular space at an equivalent rate through the lymphatic system. Albumin is also diffused into CSF and ISF compartments of the central nervous system (CNS) from blood circulation of the brain (Nicholson et al, 2000). Blood derived albumin in CSF and interstitial fluid (ISF is implicated in normal as well as many pathophysiological conditions of the brain.

4.2 Albumin in the CSF

CSF originates from choroid plexus in the ventricles. CSF flows through cisternae and subarachnoid space and finally drains through the arachnoid villi into venous blood. CSF has several important functions; it mainly helps to provide mechanical support for the brain. CSF also acts as a drainage pathway for the brain, by providing a ' sink ' into which products of metabolism or synaptic activity are diluted and subsequently removed. Also, it acts as a route of communication within the CNS, i.e., it carries hormones, nutrients and transmitters between different areas of the brain. CSF albumin is predominantly a blood derived protein and it is mainly entered from the leptomeningeal blood CSF barrier (BCSFB) or from choroid plexus BCSFB (Johanson et al, 2008). The albumin quotient (Q alb) in the CSF is approximately 30-80 % of the total protein. The altered Albumin CSF/serum ratio (QAlb) is the indicator of the dysfunction of the BCSFB. CSF serum Q alb, along with other blood derived proteins in CSF, is widely used in the diagnosis of neurological diseases (Reiber, 1998, 2003; Reiber and Peter, 2001). The exact role of albumin in CSF is not fully known, but it is proposed that albumin could be involved in the maintenance of CSF oncotic pressure, delivery of a wide range of molecules that are important for normal brain function and in the removal of some of the harmful molecules from the brain. The exact roles of albumin in CSF and in the brain function are not fully understood. However, many recent studies indicate that albumin might have a neuroprotective role via multiple mechanisms in different pathophysiological conditions.

4.3 Albumin induced neuroprotection in experimental stroke

Ischemic stroke is an acute cerebrovascular disease resulting from a transient or permanent reduction in the cerebral blood flow (CBF). It mainly occurs due to blockade of the major cerebral blood vessels by a local thrombus or an embolus. Ischemia causes reduction in the oxygen and nutrient supply to the brain areas which leads to neuronal cell damage or cell death. It can cause long-term disabilities such as muscle paralysis, cognitive deficits, language deficits, emotional deficits and even coma or death. Stroke is the third leading cause of death worldwide after coronary heart disease and cancer (Lloyd-Jones et al, 2009) and ischemic stroke comprises approximately 87 % of all types of brain strokes. The only approved treatment with intravenous fibrinolytic such as tissue plasminogen activator (tPA) within 3 h of stroke onset yields reperfusion and clinical benefits (rt -PA Stroke Study

Group, 1995; Hacke et al, 2004; Juttler et al, 2006). However, the goal is to discover a neuroprotective drug which can inhibit reperfusion injury and provide neuroprotection within a wide therapeutic window. Hemodilution is an old approach which has been investigated for many decades as a potential therapy for ischemic stroke. Infusion of dextran has been shown to increase CBF of both the normal and ischemic brain, either by decreasing blood viscosity or by vasodilation in response to diminished oxygen delivery (Wood and Kee, 1985; Korosue and Heros, 1992). Despite neuroprotective benefi ts in experimental set-ups, several clinical trials of hemodilution in ischemic stroke have nonetheless proven negative or inconclusive (Scandinavian Stroke Study Group, 1987; Italian Acute Stroke Study Group, 1988; The Hemodilution in Stroke Study Group,1989). Subsequently, albumin has emerged as an alternative hemodiluting agent to dextran owing to its volume expanding properties (Sundt et al, 1967; Little et al, 1981; Emerson, 1989). However, only recently it has been rigorously evaluated for its anti-ischemic neuroprotective efficacy. Transient focal cerebral ischemia induced by middle cerebral artery occlusion (MCAO) is the most widely used model to study molecular mechanisms of cerebral ischemia-reperfusion injury and to screen neuroprotective drugs. It is less invasive with a low rate of mortality and a low coefficient of variation in lesion size (Longa et al, 1989; Belayev et al, 1997b). In the rat MCAO model, Cole et al. reported that 5 % albumin administration at the onset of ischemia reduced ischemic brain injury as evidenced by reduced hematocrit, infarct volume and cerebral edema (Cole et al, 1990). In another study, administration of concentrated (20%) HSA (1% body weight, intravenously) to rats at the onset of recirculation induced substantial diminution of infarct volume together with a marked reduction of brain edema. Thus, it is proposed that albumin might modify water homeostasis and ultimately reduce edema of the ischemic brain (Belayev et al, 1997a). These two initial studies suggested that albumin therapy at the onset of ischemia or reperfusion induces neuroprotection.In a detailed study using magnetic resonance imaging, by means of diffusion-weighted magnetic resonance imaging (DWI), 25% Human Serum Albumin (HSA) solution (1% by body weight) administered immediately after reperfusion was associated with DWI normalization and a mitigation of pannecrotic changes within zones of residual injury at 24 h of injury. Albumin therapy lowered the hematocrit on average by 37% and raised plasma colloid oncotic pressure by 56%, improved the neurological score and reduced brain swelling throughout the 3-day survival period (Belayev et al, 1998). Similar treatment also improved local CBF as measured autoradiographically with 14C-iodoantipyrine after 1 h of recirculation (Huh et al, 1998). Using laser scanning confocal microscopy and laser Doppler perfusion imaging, it was found that a beneficial effect of albumin therapy was attributed to reversal of stagnation, thrombosis and corpuscular adherence within cortical venules in the reperfusion phase after focal ischemia (Belayev et al, 2002). It was also reported that after 1 h of reperfusion, 1.25 g/kg intravenous HSA administration increased replenishment of polyunsaturated fatty acid (PUFA) lost from cellular membranes during ischemia (Rodriguez de Turco et al, 2002). These studies collectively indicate that albumin induced neuroprotection is attributed to properties such as reversal of thrombosis, improvement in microvascular blood perfusion, reduction in brain swelling and replenishment of PUFA in brain. All these actions could indicate that actions of albumin are confined in vascular space. However, it has been shown that treatment with human albumin following 2 h of MCAO also leads to albumin extravasations

and subsequently cellular uptake. It has been observed that cortical neurons with preserved structural features had taken up human albumin. Thus, it is reasonable to speculate that treatment with human albumin could also provide direct neuronal protection (Remmers et al, 1999). For the effective treatment of ischemic stroke, treatment should be started within a narrow therapeutic window of 3 h. Moderate-dose albumin therapy (1.25 g/kg intravenously) markedly provides neuroprotection even when treatment is delayed up to 4 h after onset of ischemia (Belayev et al, 2001). Albumin treatment has also been found to be neuroprotective in other models of focal ischemia. Prompt albumin therapy improved neurological function and blood-brain barrier integrity after acute intracortical hematoma (ICH) (Belayev et al, 2005). In a model of laser-induced cortical arteriolar thrombosis, high-dose albumin therapy induced a prompt, sustained improvement in microvascular hemodynamics distal to a cortical arteriolar thrombosis (Nimmagadda et al, 2008). In acute ischemic stroke, albumin combination therapy can attenuate the deleterious effects of tPA (Tang et al, 2009). Furthermore, albumin (1.25 g/kg) treatment maintains serum albumin at a higher level and attenuates cortex and hippocampus vascular endothelial growth factor (VEGF) expression at 6 h and 1 day after MCAO. This could partially contribute to the protective effects of albumin on reduction of brain edema and infarct size in the early stage of ischemia (Yao et al, 2010). The above mentioned studies prove that in experimental transient ischemia albumin provides neuroprotection via different indirect and direct mechanisms. Albumin has been found to be effective in other models of stroke such as permanent MCAO, global ischemia induced by bilateral common carotid occlusion (BCCO) and traumatic brain injury (TBI). In permanent MCAO, rats treated with 2 g/kg/day concentrated (25%) albumin begun after 30 min of ischemia showed diminished brain edema and infarct volume up to 6 days (Matsui et al, 1993). Furthermore, albumin (1.25 and 2.5 g/kg) significantly reduced cortical and striatal infarct areas and increased cortical perfusion in the permanent ischemia model (Liu et al, 2001). In transient global ischemia, HSA-treated rats showed signifi cantly improved neurological deficits throughout a 7-day survival period along with increases in numbers of surviving CA1 hippocampal pyramidal neurons compared to saline-treated animals (Belayev et al, 1999b). In TBI, 15 min after trauma, HSA administration signifi cantly improved neurological defi cits and also significantly reduced total contusion area (Belayev et al, 1999a). These experimental trials altogether indicated significant neuroprotective roles of albumin in different models of ischemic stroke and encouraged the further development of this important molecule for possible treatment of ischemic stroke in humans.

4.4 Albumin in clinical trials for ischemic stroke

The Albumin In Acute Stroke (ALIAS) Pilot Clinical Trial was conducted during 2001 – 2005 at two clinical sites (Universities of Calgary and Miami). This study was designed to investigate the safety and tolerability of albumin therapy in acute ischemic stroke. The ALIAS Pilot Clinical Trial used a multiple-tier, open-label, dose-escalation design. Human albumin (25%) in doses ranging up to 2.05 g/kg was well tolerated by patients with acute ischemic stroke without major dose-limiting complications. Concurrent tPA therapy did not affect the safety profile of albumin (Ginsberg et al, 2006a). Also, in this pilot trial the neuroprotective efficacy of albumin was evaluated and was found to be neuroprotective after ischemic stroke (Palesch et al, 2006). Based on the encouraging results of pilot trials of

albumin, the National Institutes of Health has funded a randomized multicenter placebo-controlled effi cacy trial – the ALIAS Phase III Trial. A randomised, multicenter, double-blind, placebo controlled trial (ALIAS Phase III Trial, www.clinicaltrials. gov; NCT00235495) is currently being conducted at approximately 70 clinical sites in North America (Ginsberg et al, 2006b; Hill et al, 2011).

4.5 Direct neuroprotection by albumin: Mechanisms

The neuroprotective mechanisms of albumin in ischemic stroke and AD are largely attributed to its hemodynamic properties and binding properties. However, in different in vitro systems, albumin has been reported to possess several direct neuroprotective actions. (Figure 4)

Albumin could produce various neuroprotective actions in the intravascular compartment, cerebrospinal fluid-interstitial fluid compartment and intracellular compartment.

HSA and its N-terminal tetrapeptide DAHK can block oxidant-driven cultured neuronal injury produced by hydrogen peroxide and copper/ascorbic acid (Gum et al, 2004). Furthermore, bovine serum albumin has been found to be neuroprotective by reducing both the DNA damage and apoptosis rates in cultured cortical neurons and these effects are probably due to its antioxidant activity (Baltanas et al, 2009). Albumin has been reported to play an important role in astrocyte functions. It is shown that albumin affects metabolism of cultured astrocytes (Tabernero et al, 1999). Albumin up on transcytosis into cultured astrocytes stimulates the synthesis of neurotrophic factor oleic acid which promotes neuronal differentiation (Tabernero et al, 2002). Megalin is a receptor for albumin in astrocytes and is required for the synthesis of the neurotrophic factor oleic acid (Bento - Abreu et al, 2008). Also, this megalin induced albumin transcytosis and synthesis of

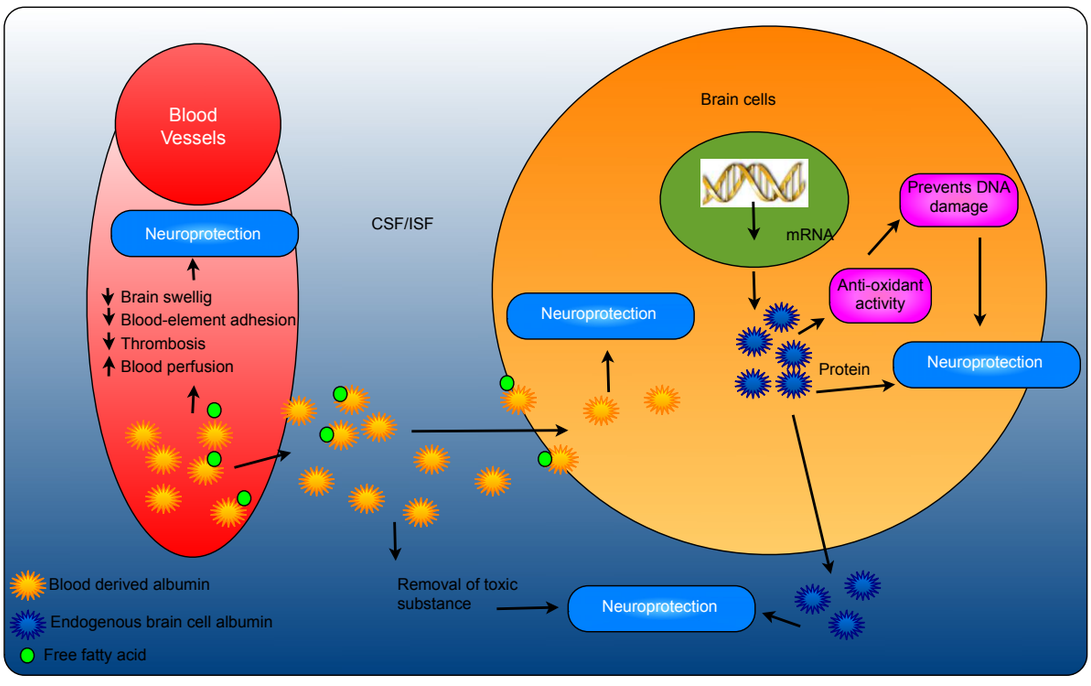

Fig. 4. Overview of possible neuroprotective mechanisms of albumin.

neurotrophic factor is dependent on caveola and the adaptor protein cytosolic adaptor protein disabled (Dab-1) in cultured astrocytes (Bento -Abreu et al, 2009). These studies indicate that albumin could play a role in neuronal differentiation and development. Albumin also induces calcium mobilisation in cultured as well as brain astrocytes (Manning and Sontheimer, 1997; Nadal et al, 1998; Hooper et al, 2005). Albumin elicits calcium entry in the microglia which promotes proliferation of the microglia (Hooper et al, 2005). Astrocyte calcium signalling caused by albumin could have important physiological and pathophysiological consequences when the blood-brain barrier breaks down and allows albumin to enter the CNS. It is reported that albumin leakage induced by blood-brain barrier breaks is followed by albumin uptake into astrocytes which is responsible for epileptogenesis in rats (Ivens et al, 2007; van Vliet et al, 2007). Albumin causes downregulation of Kir current which results in the abnormal accumulation of $[K^+]$ o and consequent NMDA-receptor dependent pathological plasticity which isresponsible for epileptogenesis (Ivens et al, 2007). Recently, it was shown that albumin activates astrocytes and microglia producing infl ammatory responses via the mitogen-activated protein kinase pathway and these effects could be involved both in the mechanism of cellular injury and repair (Ralay Ranaivo and Wainwright, 2010). Altogether these fi ndings suggest that the majority of the effects of albumin on astrocytes, microglia and neuronal cells seem to be benefi cial; however, at augmented levels it could contribute towards astrocyte dysfunction. The direct effects of albumin on neuronal and glial cells necessitate further detailed investigation in individual pathological conditions.

4.6 Endogenous albumin and neuroprotection: possible new paradigm

Although albumin is mainly synthesized in the liver, mRNA expression level of albumin has been found in many non-hepatic rat tissues such as lungs, heart, kidney and pancreas, but not in the brain (Nahon et al, 1988). Also, non-hepatic albumin expression at the protein level is rarely confirmed. A recent study suggests that human brain microglia cells can express albumin both at mRNA and protein levels; furthermore, this expression is increased by amyloid beta (Aβ) and lipopolysaccharide treatment (Ahn et al, 2008). It is suggested that enhanced levels of albumin and subsequent secretion by microglia could be implicated in A β removal from the brain (Ahn et al, 2008). We have also found upregulation of albumin at both mRNA and protein levels in ischemic rat brain. Upregulation of albumin in ischemic brain could play a neuroprotective role against altered brain functions (Prajapati et al, 2010). These results indicate that de novo synthesis of albumin also occurs in the brain tissue. However, possible intracellular and extracellular neuroprotective actions of endogenously synthesized albumin is an unexplored area and warrants further investigations.

5. Antithrombin III

Antithrombin III (ATIII) is a single-chain glycoprotein in plasma and belongs to the family of the serpins. It is synthesized in liver parenchymal cells, and it plays a central role in regulating haemostasis. When bound to glycosaminoglycans, it is an important inhibitor of several serine protease, including factors Xa, IXa, XIa, and thrombin (Bauer and Rosenberg, 1991), wich are involved in blood coagulation. Equimolar, irreversible complexes are formed between ATIII and the enzymes. Heparin and heparan sulfate glycoproteins (HSPGs) bind to multiple sites of the ATIII molecule resulting in a steric reconfiguration, thereby

increasing the interaction between ATIII and the activated enzymes. It is believed that much of the physiological inactivation of enzymes by ATIII occurs in the endothelium, mediated by heparan sulfate (Figure 5) (Mammen, 1998).

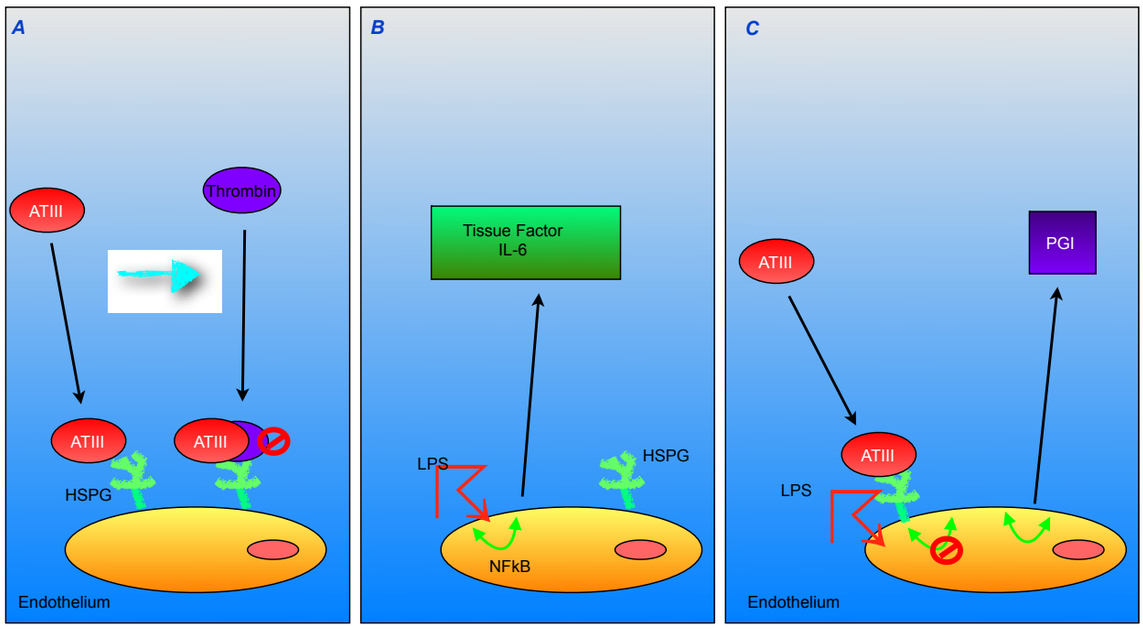

Fig. 5. Role of heparan sulfate proteoglycan (HSPG) in inhibition of thrombin, in induction of prostacyclin, and inhibition of cytokine and tissue factor release from endothelial cells by antithrombin III.

A large number of recent studies have shown that ATIII has anti-inflammatory actions, (Cuomo et al, 2007) which are independent of its effects on coagulation. These effects include the heterologous deactivation of activated leukocytes and the interaction with the endothelium, thereby reducing vessel wall transmigration and subsequent tissue and organ damage. Thus, ATIII may have two distinct and indipendent actions in patients with cerebral ischemia: (1) interference with pathologic coagulation, and (2) inhibition of inflammation.

5.1 Effects of ATIII on abnormal coagulation

The prothrombotic, proinflammatory state of stroke results in a promotion of thrombin formation and fibrin deposition at the vascular wall, as well as in the formetion of platelet-leukocyte coaggregates, leading to severe disturbance of the microcirculation, capillary leakage and tissue damage (Piazza et al, 2010). Many of the events involved in this proinflammatory state have been shown to be inhibited by ATIII. The inhibition of factor Xa by ATIII may be particularly important for protection against, and treatment of, inflammation. This activated clotting factor has a number of proinflammatory effects, including stimulation if the production of IL-6, IL-8, MCP-1, E-selectin, and the soluble adhesion molecules ICAM-1 and vascular cell adhesion molecule (VCAM)-1, wich can be experimentally blocked by ATIII (Senden et al, 1998). The proinflammatory functions of

thrombin include stimulation of neutrophil/monocyte adhesion, action as a chemotactic factor for polymorphonuclear leukocytes (Esmon, 2000), and increased expression of the recently discovered inhibitor of fibrinolysis, thrombin-activable fibrinolysis inhibitor (TAFI) (Opal, 2000). Thrombin also stimulates the increased expression of IL-8 and plays an important role in ischemia-induced leukocyte rolling and adhesion (Kaur et al, 2001; Ludwicka-Bradley et al, 2000; Rabiet et al, 1994). Thus, the ability of ATIII to inhibit the actions of both factor Xa and thrombin gives it the potential to block, in part or fully, a wide range of proinflammatory events (Seegers, 1978).

5.2 Coagulation-indipendent anti-inflammatory effects of ATIII

In the late 1980s, initial publications reported on the property of ATIII to stimulate prostacyclin release from endothelial cells independent of thrombin interaction. Although some recent studies question such a mechanism, at least in vitro, a number of papers make an in vivo contribution of prostacyclin release as part of the ATIII anti-inflammatory properties likely (Uchiba and Okajima, 2001). Independent of ATIII's anticoagulatory activities at multiple points of action, a variety of studies provide evidence for a potent anti-inflammatory ATIII effect, wich can only be induced by high ATIII plasma activities in the range of 150%-200% (Harada et al, 1999; Hoffmann et al, 2000; Okajiama and Uchiba, 1998; Uchiba et al, 1996, 1998). These anti-inflammatory ATIII actions may be mainly mediated by an interaction of ATIII with the endothelium (Hoffmann et al, 2002), thereby producing a profound increase in endothelial prostacyclin production (Figure 5C) (Yamauchi et al, 1989).

5.3 Effects of ATIII on leukocyte-endothelium interactions

ATIII expresses the ability to inhibit leukocyte rolling and adhesion, wich are hallmarks of inflammatory reactions, and have explored the mechanisms underlying these effects. Ostrovsky et al (Ostrovsky et al, 1997) showed that ATIII administration significantly reduced neutrophil rolling and adhesion to pretreatment levels in a feline mesentery ischaemia-reperfusion model. Nevière et al (Nevière et al, 2001) and Hoffmann et al (Hoffmann et al, 2000) showed that the beneficial effects resulting from ATIII's blocking of leukocyte-endothelium interactions were eliminated when indomethacin, a cyclo-oxygenase inhibitor that blocks prostacyclin production, was added to the treatment. The administration of recombinant hirudin did not result in comparable beneficial effects, supporting the thrombin-indipendent mode of action of ATIII. As a consequence of the limited activated leukocyte-endothelium interaction, the severity of subsequent capillary leakage, disturbance of microcirculation, and organ damage were significantly reduced. A report by Yamashiro et al (Yamashiro et al, 2001) suggests direct affects of ATIII on leukocytes and endothelium by demonstrating the downmodulation of P-selectin by ATIII in the LPS-stimulated endothelium, thereby diminishing leukocyte rolling and subsequent transmigration. Support of this hypothesis has also been provided by the work of Souter et al (Souter et al, 2001) showing that the addition of ATIII to LPS-treated whole blood, HUVEC, and mononuclear cells inhibited production of both IL-6 and tissue factor; recombinant hirudin, a specific thrombin inhibitor, did not reduce the production of IL-6 or tissue factor, again suggesting that the observed inhibition by ATIII was not due solely to its ability ti inhibit thrombin (figure 5B and C).

5.4 Direct effect of ATIII on leukocytes

Dunzendorfer et al (Dunzendorfer et al, 2000, 2001) have uncovered a second mechanism by which ATIII may inhibit neutrophil migration and adhesion to the endothelium; namely, heterologous deactivation of activated leukocytes by ATIII. These investigators noted that signaling in ATIII-induced neutrophil chemotaxis mimics an IL-8-induced response, ATIII inhibited migration of neutrophils towards IL-8, GRO-alpha, and fMLP; staurosporine, bisindolylmaleimide I, pertussis toxin, and an anti-CXCR1 monoclonal antibody all blocked ATIII-induced neutrophil chemotaxis. However, additional assays did not reveal binding of ATIII to CXCR1. Thus, the results of these studies are generally consistent with the conclusion that the effect of ATIII in neutrophil migration appear to involve a CXCR1-related signaling pathway, its G-proteins, and protein kinase C. Recent findings have shown that the signalling pathway activated by ATIII in leukocytes are different in neutrophils, monocytes, and lymphocytes (Dunzendorfer et al, 2001; Kaneider et al, 2001, 2002). All togheter, these experiments led to the conclusion that ATIII in circulation protects leukocytes from premature activation.

5.5 ATIII and nuclear factor-kappaB

Oelschager et al (Oelshager et al, 2001) showed that ATIII produces a dose-dependent reduction in both LPS and tissue necrosis factor (TNF)-α activation of nuclear factor-kappaB (NF-κB) in cultured monocytes and endothelial cells. Results reported by this working group and by Iampietro et al (Iampietro et al, 2000) indicate that these actions of ATIII block the increase in IL-6, IL-8, TNF, and tissue factor mRNA expression (figure 5B and C). Beyond control of coagulation, ATIII displays anti-inflammatory properties through an interaction with cells, reducing the synthesis and release of proinflammatory mediators, thereby modulating leukocyte activation and their interaction with the vessel wall. As a consequence, tissue damage and organ failure are reduced.

6. Toll-like receptors

The TLRs, so-called because of their homology to the Drosophila Toll receptor, were first characterized in mammals by their ability to recognize pathogen-associated molecular patterns such as those found in the bacterial cell wall components peptidoglycan (TLR2) and lipopolysaccharide (LPS) (TLR4), as well as viral dsRNA (TLR3), ssRNA (TLR7), and nonmethylated cytosine-guanine (CpG) DNA (TLR9). Recently it has been found that in addition to their role in pathogen detection and defense, TLRs act as sentinels of tissue damage and mediate inflammatory responses to aseptic tissue injury. Surfactant, HSP60, components of the extracellular matrix, and fibrinogen have all been shown to activate TLR4, while host HMGB1 and host mRNA and DNA are endogenous ligands of TLR2 (and TLR4), TLR3 and TLR9, respectively. TLRs, upon activation by either pathogen- or host-derived ligands, induce downstream signals that lead to cytokine and chemokine production and thereby initiate inflammatory responses. TLRs are located on antigen presenting cells such as B cells, dendritic cells, monocytes/macrophages and microglia. In addition, these receptors can be expressed by the cerebral endothelium and by cells within the brain parenchyma such as astrocytes, oligodendrocytes, and neurons (Bsibsi et al., 2002; Singh and Jiang, 2004; Jack et al., 2005; Bsibsi et al., 2006). The TLRs signal through common

intracellular pathways leading to transcription factor activation and the generation of cytokines and chemokines (Figure 6) (Vogel et al., 2003; Takeda and Akira, 2005). Each TLR family member, with the exception of TLR3, initiates intracellular signaling via recruitment of the intracellular Toll-interleukin 1 receptor (TIR)–domain-containing adaptor MyD88. When recruited to plasma membrane-associated TLRs, either directly (TLRs 5 and 11) or via the TIRAP adaptor (TLRs 1, 2, 4, 6), MyD88 enlists members of the IRAK family, including IRAK1, IRAK2, and IRAK4, to begin a process of auto- and cross-phosphorylation among the IRAK molecules. Once phosphorylated, IRAKs dissociate from MyD88 and bind TRAF6, an E3 ligase. TRAF6 in turn activates TAK1 which itself activates the IKK complex and MAPKKs. The IKK complex, composed of IKKα, IKKβ and the regulatory subunit IKKγ/NEMO, phosphorylates IκB proteins. This phosphorylation is necessary for the ubiquitination and proteosomal degradation of IκBs and the subsequent nuclear translocation of the transcription factor NFκB. Members of the MAPK family phosphorylate and activate components of the transcription factor AP-1. Together, these transcription factors induce inflammatory cytokine production (e.g. TNFα, IL1). MyD88 is also recruited to the endosomal receptors TLR7 and TLR9 again enlisting members of the IRAK family. Due to the endosomal location of the complex, the phosphorylated IRAKs are able to bind TRAF3 in addition to TRAF6. Activation of TRAF3 leads to phosphorylation, dimerization, and nuclear localization of the transcription factors IRF3, IRF5, and IRF7 with resultant type I interferon (IFN) production. Hence these endosomal TLRs are capable of signaling to NFκB, AP-1 and IRFs, resulting in a diverse genomic response. Endosomal TLR3 is unique among the TLRs because it does not signal through MyD88 but signals instead via recruitment of the Toll-interleukin 1 receptor domain-containing adaptor inducing interferon β (TRIF). TRIF enlists the non-canonical IKKs, TBK1 and IKKε, which activate IRF3. Further, TRIF recruits TRAF6 and RIP-1, which results in activation of MAPK and IKKα/β. Hence TLR3, like the other endosomal receptors, is capable of activating NFκB, AP-1 and IRFs. Of all the TLRs, only TLR4 can recruit either MyD88 (via TIRAP) or TRIF (via TRAM) and can thus induce either the pro-inflammatory cytokines TNFα and IL1 via NFκB or the anti-viral IFNβ via IRF3. The complement of TLR family members expressed by a cell depends on its identity and its activation status. Constitutive expression of TLRs within the brain occurs in microglia and astrocytes and is largely restricted to the circumventricular organs and meninges—areas with direct access to the circulation (Laflamme and Rivest, 2001; Laflamme et al., 2001; Chakravarty and Herkenham, 2005). Human and murine microglia express TLRs 1–9 and generate cytokine profiles specifically tailored by the TLR stimulated (Bsibsi et al., 2002; Olson and Miller, 2004; Jack et al., 2005). Similarly, human and murine astrocytes express multiple TLRs, with particularly prominent TLR3 expression (Bsibsi et al., 2002, 2006; Carpentier et al., 2005; Jack et al., 2005; McKimmie and Fazakerley, 2005). Microglia and astrocytes respond differently to specific TLR engagement reflective of their distinct roles in the brain. Microglia initiate robust cytokine and chemokine responses to stimulation of TLR2 (TNFα, IL-6, IL-10), TLR3 (TNFα, IL-6, IL-10, IL-12, CXCL-10, IFNβ), and TLR4 (TNFα, IL-6, IL-10, CXCL-10, IFNβ), yet astrocytes initiate only minor IL-6 responses to all but TLR3 stimulation (Jack et al., 2005). Microglia express TLR3 and TLR4 at the cell surface while astrocytes express these receptors intracellularly (Bsibsi et al., 2002). The cellular location of TLRs affects their downstream signaling cascades (Kagan et al., 2008), which may explain the different responses of these cells to TLR stimulation. The

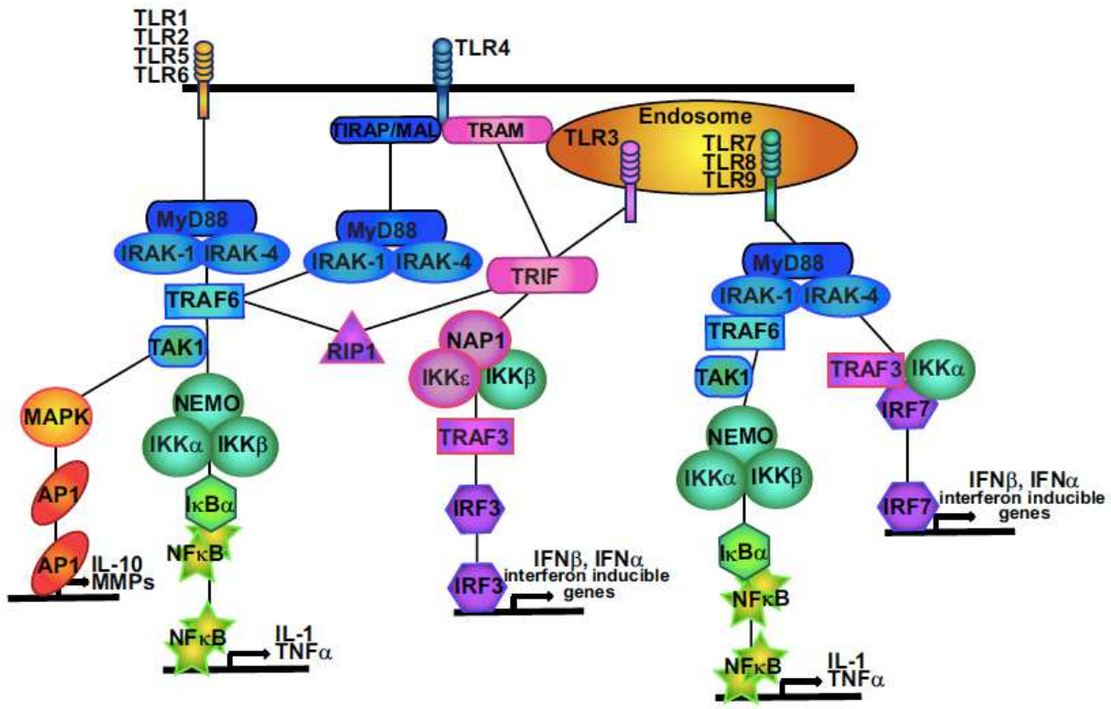

Fig. 6. TLRs signaling

inflammatory milieu also plays a critical role in regulating TLR expression. Microglia stimulated with CpG specifically up-regulate TLR9, whereas those stimulated with a synthetic TLR3 ligand suppress all TLRs except TLR3 (Olson and Miller, 2004). Similarly, astrocytes stimulated with LPS up-regulate TLRs 2 and 3 but suppress TLR4, while astrocytes exposed to RNA viruses up-regulate TLR3 and TLR9 (McKimmie and Fazakerley, 2005). Thus microglia and astrocytes initiate a layered and multifaceted response to TLR engagement. Oligodendrocytes and endothelial cells express a relatively limited repertoire of TLRs. Oligodendrocytes express TLRs 2 and 3 (Bsibsi et al., 2002), while cerebral endothelial cells constitutively express TLRs 2, 4, and 9 (Constantin et al., 2004) and increase their expression of these TLRs in response to stressful stimuli, including systemic LPS and cerebral ischemia (Singh and Jiang, 2004; Zhou et al., 2007; Ziegler et al., 2007). In response to LPS, endothelial cells up-regulate E-selectin, an NFκB-dependent molecule, and IFNβ, an IRF3-dependent molecule, indicating that these cells utilize the TLR–NFκB and the TLR–IRF3 signaling pathways (Lloyd-Jones et al., 2008). Neurons express TLR3 and generate inflammatory cytokines (TNFα, IL-6), chemokines (CCL5, CXCL10) and antiviral molecules (IFNβ) in response to dsRNA (Lafon et al., 2006; Prehaud et al., 2005). Neurons also employ TLRs in their development and differentiation. TLRs 3 and 8 are expressed on murine neurons early in development and inhibit neurite outgrowth in a MyD88- and NFκB-independent manner (Ma et al., 2006). TLR2 and TLR4 have been found on adult neural progenitor cells where they appear to elicit opposing effects. While TLR2 activation stimulates neuronal differentiation of these cells, TLR4 activation decreases proliferation and neuronal differentiation, driving these cells toward an astrocytic fate (Rolls et al., 2007). Curiously, both TLRs exert these endogenous effects in a MyD88-dependent manner,

suggesting that these molecules utilize MyD88 in distinct ways. Hence even minor alterations of these fine-tuned endogenous pathways can have profound effects on cellular responses to TLR engagement. Studies with TLR knockout mice illustrate the endogenous function of TLRs in health and disease. TLR2 and TLR4 have been shown to play detrimental roles in the development of congestive heart failure and cardiac hypertrophy, respectively, by signaling through MyD88 and NFκB (Shishido et al., 2003; Ha et al., 2005). TLR2 has additionally been found to be proatherogenic in hyperlipidemic mice (Tobias and Curtiss, 2007), and TLR4 has been shown to produce inflammatory reactions in adipose tissue and thereby mediates obesity and insulin resistance (Tsukumo et al., 2007; Davis et al., 2008). Conversely, TLR2 and TLR4 activation by hyaluronic acid protects lung tissue from non-infectious injury (Jiang et al., 2006), and TLR4 has been shown to help maintain lung integrity, and prevent the development of emphysema, by modulating oxidant generation (Zhang et al., 2006). The effects of endogenous TLR stimulation are clearly varied, depending on the cell and tissue type in which the receptors are found and on the disease process in which they are involved. The overwhelming and generally damaging inflammatory response of TLRs to aseptic tissue injury may be a consequence of TLR evolution in response to pathogens. In the setting of pathogen invasion, an inflammatory deluge may be the most effective means to clear microorganisms. The activation and influx of leukocytes, with the concomitant release of free radicals and tissue-destroying enzymes, assails not only the invading pathogen but any host cells that harbor the pathogen. However, when this same powerful response is co-opted by the host to clear and resolve tissue damage, it can destroy the very cells it is meant to save. This damage promoting characteristic is prominently observed following brain ischemia, where inflammation plays a critical role in both injury progression and resolution.

6.1 TLRS and ischemic damage

A significant portion of the damage associated with stroke injury is due to the resultant inflammatory response. This aspect of the innate immune response is exemplified by the fact that some anti-inflammatory strategies have been shown to ameliorate ischemic damage (Relton et al., 1996; Hara et al., 1997; Spera et al., 1998). The inflammatory
response to stroke is initiated by the detection of injury associated molecules by local cells such as microglia and astrocytes. The response is further promoted by infiltrating neutrophils and macrophages, resulting in the production of inflammatory cytokines, proteolytic enzymes, and other cytotoxic mediators. In the mouse, leukocytes and brain cells (microglia, astrocytes and neurons) express TLRs (Zarember and Godowski, 2002; Olson and Miller, 2004). Hence, injury-associated molecules such as HSP60 and HMGB1 may act as endogenous ligands for TLRs, thereby initiating the damaging inflammatory response to stroke. It is increasingly clear that TLRs do in fact play a role in ischemic damage (Fig.). The pathogenic role of TLRs in ischemic processes was first demonstrated in a mouse model myocardial ischemia/reperfusion injury, because mice lacking functional TLR4 incur less damage than wild type mice (Oyama et al., 2004). Since then, TLR2 has also been shown to cause dysfunction following cardiac ischemia and both have been shown to exacerbate renal ischemic damage, in a MyD88-dependent and a MyD88-independent manner (Sakata et al., 2007; Shigeoka et al., 2007). However, the particular pathway responsible for the damaging effects of TLR activation may differ depending on the cell type or organ affected as TLR4

worsens ischemic damage following liver transplant in a MyD88-independent, IRF3 dependent fashion (Zhai et al., 2004; Shen et al., 2005). Importantly, TLR2 and TLR4 have been shown to play a role in cerebral ischemic damage. Mice lacking either functional TLR2 or TLR4 are less susceptible to transient focal cerebral ischemia/reperfusion damage, demonstrating smaller infarcts than wild type controls (Cao et al., 2007; Lehnardt et al., 2007; Ziegler et al., 2007). Further, mice lacking TLR4 incur less damage following global cerebral ischemia and permanent focal ischemia (Caso et al., 2007; Hua et al., 2007). The TLR endogenous ligands HSP 60, HSP70 and HMGB1 are found in the brain following injury (Kinouchi et al., 1993; Faraco et al., 2007; Lehnardt et al., 2008). Hence these molecules may activate TLR2 and TLR4 within the brain itself, leading to the generation of inflammatory mediators such as TNFα, IL1, IL6, and iNOS, all known to be associated with stroke damage.

6.2 TLRS and neuroprotection

In contrast to the detrimental role of TLRs in response to ischemia, stimulation of these receptors prior to ischemia provides robust neuroprotection. TLR4-induced tolerance to cerebral ischemia was first demonstrated with low dose systemic administration of LPS (endotoxin), a cell wall component of gram-negative bacteria, which caused spontaneously hypertensive rats to become tolerant to subsequent ischemic brain damage induced by middle cerebral artery occlusion (MCAO) (Tasaki et al., 1997). Since then, LPS-induced tolerance to brain ischemia has been demonstrated in a mouse model of stroke and in a porcine model of deep hypothermic circulatory arrest (Rosenzweig et al., 2004; Hickey et al., 2007) (for additional information on the dual effects of neuro-immune crosstalk, please refer to Kerschensteiner et al., in this issue). Neuroprotection induced by LPS is time and dose dependent. Tolerance appears by 24 h after LPS administration and extends out to 7 days but is gone by 14 days (Rosenzweig et al., 2007). Protective doses of LPS appear to depend on the animal model of stroke and the route of systemic administration, ranging from 0.02–1 mg/kg (Tasaki et al., 1997; Ahmed et al., 2000; Bordet et al., 2000; Furuya et al., 2005; Hickey et al., 2007; Kunz et al., 2007; Rosenzweig et al., 2007). Tolerance induction has been shown to require new protein synthesis and a modest inflammatory response, as it can be blocked by prior administration of cycloheximide or dexamethasone (Bordet et al., 2000). Specifically, TNFα has been implicated as a mediator of LPS-induced ischemic tolerance because inhibition of TNFα systemically (Tasaki et al., 1997) or within the brain (Rosenzweig et al., 2007) blocks neuroprotection, and mice lacking TNFα fail to be protected by LPS preconditioning (Rosenzweig et al., 2007). In addition to its neuroprotective effects, LPS preconditioning has vasculoprotective efficacy. Nitric oxide appears to play a critical role in the protective effects of LPS. Mice lacking iNOS expression fail to be protected by LPS pretreatment (Kunz et al., 2007), and eNOS expression within the brain is directly correlated to the time window of LPS-induced neuroprotection (Furuya et al., 2005). LPS pretreatment has further been shown to prevent the impairment of endothelial and smooth muscle relaxation normally induced by ischemia/reperfusion injury (Bastide et al., 2003), resulting in normalization of cerebral blood flow in peri-infarct regions lasting out to 24 h after MCAO (Dawson et al., 1999; Furuya et al., 2005). LPS-induced ischemic protection requires an inflammatory response prior to the ischemic event, yet protection occurs through modulation of the inflammatory response following ischemia. Rosenzweig et al. (2004) have

shown that LPS preconditioning changes the response of circulating leukocytes to stroke, attenuating stroke-induced neutrophilia, lymphopenia, and monocyte activation. This altered inflammatory response extends into the brain itself. LPS preconditioning attenuates activation of microglia after stroke and reduces neutrophil infiltration into the ischemic hemisphere. Hence, LPS-induced preservation of microvascular function following MCAO may be due to suppressed lymphocyte adhesion to activated endothelium, either by TNFα-induced suppression of endothelial activation and adhesion molecules (Ginis et al., 1999; Ahmed et al., 2000) or by prevention of cellular inflammatory responses to ischemia (Rosenzweig et al., 2004). One hallmark of LPS preconditioning is suppression of cytotoxic TNFα signaling following stroke. Mice that have been preconditioned with LPS prior to ischemia display a pronounced suppression of the TNFα pathway following stroke, as evinced by reduced TNFα in the serum, decreased levels of cellular TNFR1, and enhanced levels of neutralizing soluble-TNFR1. These mice are thus protected from the cytotoxic effects of TNFα after cerebral ischemia (Rosenzweig et al., 2007). Collectively, these mechanisms lead to a muted TNFα response to ischemic injury and increased cell survival. Recently a new TLR ligand has been shown to induce tolerance to brain ischemia. As with TLR4 and LPS, stimulation of TLR9 by systemically administered CpG oligodeoxynucleotides induces robust protection against brain ischemia in a time and dose dependent manner. CpG pretreatment protects neurons in both in vivo and in vitro models of stroke (Stevens et al., 2008). Notably, the protection afforded by CpG depends on TNFα, as systemic CpG administration acutely and significantly increases serum TNFα, and TNFα knockout mice fail to be protected by CpG preconditioning. Similarities among the known TLR signaling pathways and their shared ability to induce TNFα, itself a potent preconditioning stimulus, suggest that stimulation of TLR4 and TLR9 may induce ischemic tolerance by similar means. The neuroprotective potential of other TLRs has yet to be explored, but this family of molecules may be a rich source of therapeutic targets. The finding that TLRs are mediators of ischemic injury provides insight into the potential mechanisms of LPS- and CpG-induced neuroprotection. In fact, TLR-induced tolerance to subsequent ischemia may occur by the same mechanisms that govern a very similar phenomenon—that of LPS-induced tolerance to subsequent LPS exposure. The latter phenomenon is known as "endotoxin tolerance" and occurs when pretreatment with a low dose of LPS renders cells or whole animals tolerant to the normally detrimental effects of a second, higher dose of LPS. Cells that are tolerant to LPS are defined by their inability to generate TNFα in response to TLR4 activation. Upon TLR4 ligation, LPS tolerant cells, unlike naive cells, do not recruit MyD88 to TLR4, and fail to activate IRAK-1 and NFκB (Medvedev et al., 2002). The TLR4–NFκB signaling axis becomes decommissioned following a primary exposure to LPS via an elaborate negative feedback loop that involves known inhibitors of TLR signaling. Among those inhibitors are Ship-1, which prevents TLR4-MyD88 interaction, IRAK-M, a non-functional IRAK decoy, and TRIM30α, which destabilized the TAK1 complex (Kobayashi et al., 2002; Sly et al., 2004; Shi et al., 2008). Thus, subsequent signaling of TLR4 to NFκB is blocked and inflammatory cytokine production is suppressed. Conversely, secondary exposure causes enhanced IFNβ release, suggesting increased signaling via the TLR4-IRF3 axis (Broad et al., 2007). Thus, pretreatment with LPS causes cells to switch their transcriptional response to TLR4 stimulation by enhancing the IRF3- induced cytokine IFNβ and suppressing the NFκB-induced cytokine TNFα. Similar to

endotoxin tolerance, priming TLR9 with its ligand, CpG, induces a state of hypo-responsiveness to subsequent challenge with CpGs (Dalpke et al., 2005). Interestingly, cross-tolerance between the two receptors has also been reported, as ligands for TLR9 induce tolerance against a subsequent challenge with a TLR4 ligand (Bagchi et al., 2007; Broad et al., 2007). CpG-pretreated cells not only produce less TNFα when secondarily challenged with LPS, they also produce significantly higher levels of IFNβ (Broad et al., 2007). Together, the aforementioned studies suggest the intriguing possibility that TLR stimulation prior to stroke may reprogram ischemia-induced TLR activation (Fig.). Specifically, administration of LPS or CpG may activate TLR4 and TLR9, respectively, causing a small inflammatory response, with an initial rise in TNFα. Cells would then regulate their inflammatory response through expression of negative feedback inhibitors of the TLR4–NFκB signaling axis that remain present when cells are subsequently exposed to endogenous TLR ligands generated from ischemia-injured tissue. Within this new cellular environment, stimulated TLRs such as TLR2 and TLR4 would be unable to activate NFκB-inducing pathways. Because of this, stroke-induced TLR2 signaling may be blocked completely leading to reduced injury, and stroke-induced TLR4 signaling would shift from NFκB induction to IRF3 induction (Fig.). Suppression of NFκB induction would be expected to protect the brain, as mice lacking the p50 subunit of NFκB suffer less cerebral ischemic damage than wild type mice (Schneider et al., 1999). Enhancement of IRF signaling would also be expected to protect the brain, as IFNβ, a downstream product of IRF3 induction, has been shown to act as an acute neuroprotectant (Liu et al., 2002; Veldhuis et al., 2003a). IFNβ, best known for its anti-viral effects, has potent anti-inflammatory activities as well. Several studies have shown that IFNβ can stabilize the blood–brain barrier, potentially by reducing matrix metalloprotease production by activated glia (Veldhuis et al., 2003b; Kraus et al., 2004; Liuzzi et al., 2004). Similarly, it has been shown to inhibit monocyte migration across human brainderived endothelial cells (Seguin et al., 2003) and reduce cellular infiltration into damaged brain regions (Veldhuis et al., 2003b). On a cellular level, IFNβ has been shown to reduce reactive oxygen species (Lopez-Collazo et al., 1998; Stewart et al., 1998; Hua et al., 2002), suppress inflammatory cytokine production and induce IL-1Ra (Bosca et al., 2000; Palmer et al., 2004), promote nerve growth factor production by astrocytes (Boutros et al., 1997) and protect neurons from toxicity induced by activated microglia (Jin et al., 2007). In addition, systemic administration of IFNβ has been shown to reduce infarct damage in rat and rabbit models of ischemic stroke (Liu et al., 2002; Veldhuis et al., 2003a). Therefore, in the setting of LPS preconditioning, upregulation of this cytokine following stroke would be expected to contribute to neuroprotection. IFNβ may not be the only neuroprotective molecule downstream of IRF signaling. TLR3 signals exclusively through the TRIF-dependent pathway and stimulation of TLR3 in human astrocyte cultures induces the expression of several neuroprotective molecules such as brainderived neurotrophic factor, neurotrophin 4, pleiotrophin, and TGFβ2 (Bsibsi et al., 2006), all of which have been implicated in endogenous neuroprotection (Yeh et al., 1998; Endres et al., 2000; Zhang et al., 2005). Astrocytic TLR3 stimulation also results in production of the anti-inflammatory cytokine IL-10 (Bsibsi et al., 2006). Conditioned media from these cultures enhance neuronal survival and suppress astrocyte growth in slice cultures. Interestingly, LPS stimulation of macrophages has been shown to upregulate TLR3 expression (Nhu et al., 2006), inviting the possibility that LPS preconditioning may upregulate TLR3 in the brain, further enhancing

stroke-induced IRF signaling. We suggest that pretreatment with TLR ligands reprograms the brain's response to ischemia and alters endogenous stroke-induced TLR signaling by suppression of the NFκB-inducing pathway and upregulation of the IRF-inducing pathway. Reprogramming causes a finely controlled shift in the balance of proinflammatory and antiinflammatory cytokines, and represents an endogenously orchestrated mechanism that protects the organism from additional damage. We further suggest that reprogramming of endogenous TLR signaling, with the subsequent generation of neuroprotective type I IFNs, is a unifying property of the neuroprotected phenotype. The brain has evolved numerous mechanisms that allow it to withstand the shortage of energy and the oxidative stress caused by ischemia. This tolerant state can be induced by prior exposure to LPS or CpG, or by prior exposure to other non-damaging (i.e. sub-threshold) noxious stimuli. For example, mild exposure to ischemia, excitotoxic stimuli, or inflammatory mediators can precondition the brain to better tolerate a subsequent injurious ischemic event. These mild preconditioning exposures herald impending danger and, as such, induce endogenous protective strategies in anticipation of injury. Though the final outcome of tolerance induction is the same—protection of brain tissue from ischemic injury—the effector mechanisms employed by the brain are as diverse as the preconditioning stimuli that induced them. In fact, the phenotype of neuroprotection may be specifically tailored by the nature of the preconditioning stimulus (Stenzel-Poore et al., 2007). For example, preconditioning events that deprive the brain of oxygen or glucose for a short time lead to conservation of energy regulation and mitochondrial integrity during the injurious ischemic episode (Stenzel-Poore et al., 2003; McFalls et al., 2006). Further, as we have described above, preconditioning events that invoke a small inflammatory response lead to altered inflammatory responses to damaging ischemia (Rosenzweig et al., 2004, 2007). It should be emphasized that although significant overlap exists in the cellular processes induced by these diverse stimuli, the pathways that dominate each response are distinct. The first demonstration that a short period of oxygen deprivation could protect the brain from a subsequent extended period of hypoxia occurred in 1943 (Noble, 1943). Since then, hundreds of studies have been undertaken to better understand the underlying mechanisms of "ischemic preconditioning." Though several endogenously protective pathways are induced by the initiating ischemic event, one particular theme is emerging—that of mitochondrial maintenance and energy conservation (Dirnagl and Meisel, 2008). The priming ischemic episode appears to induce cellular pathways that protect mitochondria against stroke induced deficits in the electron transport chain (Dave et al., 2001). These pathways protect mitochondrial membrane potential (Wu et al., 2004), preserve mitochondrial cytochrome c (Zhan et al., 2002), increase mitochondrial sequestration of Ca^{+} and increase Ca^{+}-ATPase activity. In addition, ischemic preconditioning appears to suppress molecules that regulate ion channels, leading to channel arrest—i.e. reduction in ion permeability through the plasma membrane—which has been shown to reduce the amount of ATP required to maintain ionic homeostasis (Buck and Hochachka, 1993; Stenzel-Poore et al., 2003). Finally, a decrease in the overall cellular metabolic rate limits the stressful effects of oxygen deprivation. The pre preconditioning stimulus suppresses the expression of genes involved in protein turnover, proteasomal degradation, and energy metabolism (Stenzel-Poore et al., 2003). Although ischemic preconditioning has also been shown to help maintain protein structure and function and to suppress the damaging inflammatory response to

stroke, it is increasingly clear that sustaining mitochondrial integrity and conserving energy are important mechanisms driving endogenous ischemic tolerance. Several studies have shown that the priming ischemic event induces HSP70 within the brain (Truettner et al., 2002). In addition to its role in stabilizing protein structure, HSP70 acts as an endogenous ligand of TLR4. In fact, extracellular HSP70 has been shown to induce endotoxin tolerance (Aneja et al., 2006). Hence TLRs may be stimulated in the course of ischemic preconditioning, resulting in a reprogrammed TLR response to subsequent injurious ischemia. One of the molecular consequences of reprogrammed TLR signaling is an increase in IFNβ. Notably, IFNβ has been shown to aid in the maintenance of mitochondrial integrity. For example, treatment of astrocytes with IFNβ prevents neuronal mitochondrial respiratory chain damage (Stewart et al., 1998) and reduce IFNβ induced nitric oxide synthase (Stewart et al., 1997). Thus reprogrammed TLR signaling may help shape the phenotype of ischemia-induced tolerance The phenomenon of inflammation-induced cross-tolerance to ischemia is not limited to LPS, but extends to TNFα as well. Nawashiro et al. (1997) were the first to demonstrate that intracisternal administration of TNFα protects the brain from subsequent ischemic challenge. This protection is correlated to a decrease in CD11b immunoreactivity, suggesting a decrease in the inflammatory response to ischemia in the setting of preconditioning. Consistent with this observation, TNFα pretreatment of astrocytes and endothelial cells, through its signaling intermediate ceramide, produces a state of hypo-responsiveness as pretreated cells fail to upregulate ICAM-1 during subsequent hypoxia (Ginis et al., 2002). The decrease in ICAM-1 does not reflect global cellular suppression, but instead signifies a reprogrammed genomic response to stroke, as the hypoxiainduced expression of cytoprotective MnSOD is not affected by preconditioning. Evidence for a reprogrammed genomic response to ischemia is supported by the observation that TNFα preconditioning prevents hypoxia-induced phosphorylation of the proinflammatory transcription factor component NFκBp65, thereby preventing its interaction with the transcriptional activator p300. Taken together, these data indicate that pretreatment with TNFα reprograms the cellular environment and hence alters inflammatory reactions in response to ischemia. Just as TNFα can induce tolerance to subsequent ischemic exposure, it can induce tolerance to subsequent LPS exposure (Porter et al., 1998; Ferlito et al., 2001; Murphey and Traber, 2001). Hence TNFα preconditioning has the potential to induce a state of cross-tolerance to TLR ligands, and thereby reprogram the TLR response to stroke. IFNβ has been shown to cause many of the effects observed in TNFα-induced ischemic tolerance, such as suppression of inflammatory cytokine production, including TNFα itself, and reduction of cellular infiltration into ischemic brain regions (Veldhuis et al., 2003a). Together, these studies suggest that multiple preconditioning stimuli may cause a reprogrammed TLR response to stroke. IFNβ, produced secondary to this reprogrammed response, may aid in maintaining mitochondrial stability and in dampening the inflammatory responses to injurious ischemia.

7. The receptor for Advanced Glycation End Products (RAGE)

Advanced glycation end products (AGEs) are nonenzymatical adducts of proteins, lipids, and nucleic acids which form in a time-dependent manner in a pro-oxidant environment, especially when target molecules turnover slowly and the level of aldoses is elevated

(Schmidt et al, 1995; Vlassara et al, 1994; Bierhaus et al, 1998; Baynes, 2003; Thornalley, 1998; Brownlee, 2000). Glycation of macromolecules was originally thought to mark senescent proteins for subsequent degradation by macrophages. Receptors binding AGEs were regarded as scavenger receptors involved in AGE disposal and cell regeneration, and defective clearance of such modified proteins was believed to be important in aging and diseases with accelerated AGE-formation, such as diabetes or atherosclerosis (Vlassara et al,1994, 1985). However, when the receptor for AGEs (RAGE) was cloned and first characterized (Neeper et al,1992; Schmidt et al,1992, 1994) it turned out that binding of AGEs to RAGE did not accelerate their clearance and degradation. Rather, ligand–receptor interaction induced sustained post-receptor signaling, including activation of p21ras, MAP kinases, and the NF-κB pathway (Lander et al, 1997; Basta et al, 2002; Bucciarelli et al, 2002). Thus, the concept of RAGE as a scavenger/clearance receptor has to be revised and extended.

7.1 RAGE: Structure and ligand recognition

RAGE is a member of the immunoglobulin superfamily of cell surface molecules (Schmidt et al, 1993; Sugaya, 1994). The gene is localized on chromosome 6 near the HLA locus in the vicinity of the MHCIII complex in humans and mice, in close proximity to the homeobox gene HOX12 and the human counterpart of the mouse mammary tumor gene int-3 (Malherbe et al, 1999). The receptor is composed of three immunoglobulin-like regions: one "V"- type domain and two "C"-type-domains, a short transmembrane domain, and a 43-amino acid cytoplasmic tail (Neeper et al, 1992; Schmidt et al, 1994; Lander et al, 1997). While the "V-type" domain confers ligand binding, the cytoplasmic tail is critical for intracellular signaling. Shortly after RAGE was recognized as a receptor for AGEs, it became evident that a number of other ligands also interacted with the receptor (Bucciarelli et al, 2002; Schmidt et al, 2001; Du Yan et al, 1997; Yan et al, 1996, 2000). Structural analysis of ligand–RAGE interaction revealed that the receptor recognized three-dimensional structures, such as β-sheets and fibrils, rather than specific amino acid sequences (i.e., primary structure) (Bucciarelli et al, 2002; Schmidt et al, 2001). In addition to AGEs, RAGE binds amyloid-β peptide (accumulating in Alzheimer's disease) (Du Yan et al, 1997; Yan et al, 2000) and amyloid A (accumulating in systemic amyloidosis). Further, ligands of RAGE are S100/calgranulins, a family of closely related calcium-binding polypeptides that accumulate extracellularly at sites of chronic inflammation (Hofmann et al, 1999; Marenholz et al, 2004). Another proinflammatory ligand of RAGE is the DNA binding protein HMGB1 (amphoterin), which is released by cells undergoing necrosis (Hori et al, 1995; Wang et al, 1999; Anderson and Tracey, 2003; Treutiger et al, 2003). Besides binding ligands actively participating in chronic inflammatory and immune responses, RAGE also interacts with surface molecules on bacteria (Chapman et al, 2002), prions (Sasaki et al, 2002), and leukocytes (Chavakis et al, 2003). Thus, RAGE is much more than a receptor for AGEs; it has a broad repertoire of ligands, which share the propensity to accumulate in tissues during aging, chronic degenerative diseases, inflammation and the host response (Tretiger et al,2003). Therefore, RAGE should be considered a pattern recognition receptor (PRR) (Schmidt et al, 2001; Chavakis et al, 2003; Liliensiek et al, 2004; Gordon, 2002), and potential similarities to members of the family of Toll-like receptors should be considered (Akira et al, 2001).

7.2 RAGE-mediated NF-κB activation

Engagement of RAGE results in intracellular signaling which leads to activation of the proinflammatory transcription factor NF-κB, the latter rapidly activated as part of the first line of cellular defense (Bierhaus et al, 2001). In resting cells, NF-κB resides in the cytoplasm in its inactive form bound to the inhibitor molecule IκBα (Barnes and Karin, 1997). Upon activation, IκBα is rapidly phosphorylated and degraded, resulting in release and translocation of NF-κB (preferentially the NF-κB-heterodimer p50/p65) into the nucleus. Subsequent to nuclear translocation, NF-κB binds to decameric DNA sequences and activates transcription of NF-κB regulated target genes, such as cytokines, adhesion molecules, prothrombotic and vasoconstrictive gene products, RAGE itself, and IκBα (Barnes and Karin, 1997; Li and Schmidt, 1997; Bierhaus et al, 2000). A number of anti-apoptotic genes, including Bcl-XL, Bcl-2, and the Bcl-2 homologues A1, are also under control of NF-κB. NF-κB activation therefore provides a rapid and sensitive cellular response in the absence of new protein synthesis, which promotes cellular survival. One unique feature of RAGE-mediated NF-κB activation is the prolonged time course which appears to overwhelm endogenous autoregulatory feedback inhibition loops (Bierhaus et al, 2001). NF-κB activation subsequent to ligation of RAGE is initiated by the degradation of IκBα and IκBβ, followed by new synthesis of NF-κBp65 in the presence of newly synthesized IκBβ. De novo synthesis of p65 mRNA results in a constantly growing pool of excess transcriptionally active NF-κBp65. In contrast, the amount of newly synthesized IκBα is not sufficient to retain NF-κBp65 in the cytoplasm. In addition, newly synthesized IκBβ has been shown to be hyperphosphorylated, thereby sequestering newly synthesized NF-κB from IκBα (Thompson et al, 1995; Johnson et al, 1996)? Thus, new synthesis of IκBβ might further promote RAGEdependent sustained NF-κB activation. Since, in turn, RAGE expression is induced by NF-κB (Li and Schmidt, 1997), sustained activation of NF-κB results in upregulation of the receptor and further ensures maintenance and amplification of the signal.

7.3 AGEs and RAGE affect cellular defense mechanisms

Besides activating proinflammatory responses, RAGE downregulates cellular defense mechanisms. Ligation of RAGE by AGEs results in the suppression of reduced glutathione (GSH) and ascorbic acid levels and thereby contributes to increased intracellular oxidant stress (Lander et al, 1997; Bierhaus et al, 1997). Depletion of glutathione accounts for diminished glyoxalase-1 recycling and decreased in situ activity of glyoxalase-1 (Thornalley,1998). Glyoxalase-1, in turn, is required to catalyze the conversion of reactive, acyclic alpha-oxoaldehydes into the corresponding alpha-hydroxyacids (Degenhard 1998; Thornalley et al,1999). Since alpha-oxoaldehydes, such as methylglyoxal, represent the largest pool of reactive intracellular AGEs, glyoxalase-1 has an important role in reduction of the cellular AGE load. Consistent with this concept, in vitro experiments with cultivated endothelial cells have demonstrated that glyoxalase-1 overexpression prevents intracellular AGE formation (Shinohara et al,1998). Studies in the model organism Caenorhabditis elegans have recently confirmed that overexpression of glyoxalase-1 not only prevents AGE formation, but also protects the animals from deleterious effects of oxidant stress, as evidenced by increased longevity (Morcos et al,2004). These observations imply that engagement of RAGE not only results in increased cellular activation, but also in reduction of AGE detoxifying mechanisms.

7.4 RAGE and neuroprotection

RAGE transcription is controlled by several transcription factors, including SP-1, AP-2, NF-κB, and NF-IL6 (Li and Schmidt, 1997). RAGE expression occurs in both a constitutive and inducible manner, depending on the cell type and developmental stage (Hori et al,1995; Brett et al,1993). Whereas RAGE is constitutively expressed during embryonic development, its expression is downregulated in adult life. However, known exceptions are skin and lung, which constitutively express RAGE throughout life. Most other cells, including monocytes/macrophages, endothelial cells, smooth musclecells, fibroblasts, and neuronal cells, do not express significant amounts of RAGE under physiological conditions but can be induced to express RAGE in situations where either ligands accumulate and/or transcription factors regulating RAGE are activated (Basta et al, 2002; Bucciarelli et al, 2002; Hanford et al, 2004; Akira et al, 2001; Li et al,2004; Sorci et al, 2004a, 2004b; Cortizo et al, 2003; Shanmugam et al, 2003; Ishihara et al, 2003). Due to its ability to sustain cellular activation, RAGE has the potential to function as a master switch capable of converting a transient proinflammatory response, evoked by an inflammatory stimulus into sustained cellular dysfunction (Schmidt et al, 2001; Bierhaus et al, 2001). The majority of cellular stressors induce both the formation of reactive oxygen species (ROS) and transient activation of NF-κB (Yeh et al, 2001; Taguchi et al, 2000; Huttunen et al, 19999; Huang et al, 2001; Wautier et al, 2001). In addition, inflammatory cells directly release RAGE ligands, such as S100/calgranulins and HMGB-1 (Kokkola et al, 2005). The myeloperoxidase system of human phagocytes generates N" -(carboxymethyl)lysine, a highly reactive AGE and RAGE-ligand, at sites of inflammation (Anderson et al, 1999; Kislinger et al, 1999). High glucose concentrations promote AGE formation inside and outside cells (Brownlee, 2000; Schiekofer et al, 2003). Such time-dependent formation of AGE might also play a role in the expression of binding sites for amyloid peptides (Yan et al, 2000). In turn, RAGE has been shown to mediate transport of pathophysiologically relevant concentrations of amyloid-β peptide into the CNS (Mackic et al, 1998). Thus, stimuli initially inducing oxidant stress and NF-κB activation have the potential to activate RAGE and thereby sustain NF-κB-dependent gene expression. Activation of NF-κB results in increased RAGE expression and increases the number of ligand binding sites, thereby prolonging NF-κB activation (Schmidt et al, 2001; Bierhaus et al, 2001). Frequently, the biology of RAGE coincides with settings in which ligands of the receptor accumulate, especially in a proinflammatory environment such as diabetes mellitus, atherosclerosis, neurodegenerative disorders, rheumatoid arthritis, chronic renal disease, and inflammatory bowl disease (Basta et al, 2002; Schmidt et al, 2001; Lalla et al, 2001; Wendt et al, 2003; Bierhaus et al, 2004; Sakaguchi et al, 2003; Kislinger et al, 2001; Drinda et al, 2004; Chen et al, 2004; Goosa et al, 2001). To better understand the role of RAGE in these pathophysiological situations, interaction of ligands with cell surface RAGE was intercepted using soluble RAGE (sRAGE). Soluble RAGE is a truncated form of the receptor comprising the extracellular domain and thereby functions as a decoy that prevents ligands from interacting with cell surface receptor. Application of sRAGE in vitro and in vivo resulted in an effective blockade of RAGE, according to a decoy mechanism, in a range of animal models (Hudson et al, 2003; Lue et al, 2001; Arancio et al, 2004; Constien et al, 2001). sRAGE prevented development of micro- and macrovascular diseases in rodents, suggesting a key role for RAGE in the development of chronic vascular disorders. Moreover, sRAGE efficiently reduced late complications of experimental diabetes in both autoimmune (Chen et al, 2004) and streptozotocin induced diabetes (Wendt et al, 2003; Bierhaus et al,

2004), restored delayed wound healing (Goosa et al, 2001), protected rodent from tumor metastases and growth of primary tumors (Taguchi et al, 2000), and improved the outcome of experimental colitis (Hofmann et al, 1999). sRAGE and anti-RAGE F(ab')2-fragments suppressed abnormal findings associated with Alzheimer's-like pathology in transgenic rodent models (Lue et al, 2001; Arancio et al, 2004) and reduced the transport of amyloid-β-peptide across the blood-brain barrier (Mackic et al, 1998) . Since most of the data obtained with sRAGE were confirmed by application of neutralizing antibodies to the receptor and/or transfection with plasmids overexpressing dominant negative RAGE, the receptor has been suggested as a potentially effective therapeutic target (Hudson et al, 2003). At the same time, it seemed unlikely that RAGE could mediate so many deleterious effects in such diverse models of disease. Since RAGE has properties of a PRR, binding to a variety of ligands, the promising effects observed with sRAGE might not only result from intercepting the interaction of ligands with cell surface RAGE, but possibly with other receptors. For example, S100 proteins and HMGB1 certainly do not exclusively bind to RAGE. These ligands also recognize other cellular structures (Robinson et al, 2002; Erlandsson et al, 2004). In order to test the potential impact of RAGE blockade and to further define a potential role of RAGE in diabetic complications and chronic inflammatory disease, homozygous RAGE-deficient mice (RAGE−/− mice) and mice with tissue-specific RAGE expression (tie2-RAGE and tie2-RAGE°—RAGE−/−) have been made (Constien et al, 2001). These mice are viable and display normal reproductive fitness without any striking phenotype (Wendt et al, 2003; Bierhaus et al, 2004; Sakaguchi et al, 2003). Induction of diabetes in these mice confirmed that RAGE contributes, at least in part, to the development of diabetic complications. Diabetic nephropathy, characterized by renal enlargement, glomerular hypertrophy, albuminuria, and mesangial expansion, was significantly increased in diabetic mice overexpressing RAGE in the vasculature, but was reduced in RAGE−/−mice (Yamamoto et al, 2001). Similar changes were observed in diabetic neuropathy. Whereas diabetic mice overexpressing RAGE showed an increase in functional deficits, such as delayed motor nerve conduction velocity (Yajima et al, 2004), RAGE−/− mice were partially protected from diabetes-induced loss of neural function (Bierhaus et al, 2004). Neointimal expansion in RAGE−/− mice was significantly suppressed compared with that observed in wildtype littermates using a femoral artery denudation protocol to induce arterial injury (Sakaguchi et al, 2003). Remarkably, in each of these models (diabetic nephropathy, neuropathy, arterial restenosis, etc.), protection from development of pathology was more profound in wild-type mice treated with sRAGE than in RAGE−/− mice. In diabetic neuropathy, for example, administration of sRAGE to diabetic wild-type animals completely restored pain perception, whereas diabetic RAGE−/− mice were only partly protected from loss of pain perception. These observations suggest that ligands sequestered by sRAGE are likely to interact with cellular structures different from RAGE and are also involved in perturbation of pain perception. The absence of a developmental phenotype in RAGE−/− mice and the possibility that RAGE might impact on multiple chronic disease states have largely focussed attention away from physiologic roles of the receptor. So far, only a few reports have suggested that RAGE expression might contribute to developmental paradigms, based on in vitro studies. For example, in axonal sprouting which accompanies neuronal development, RAGE–HMGB1 interaction may contribute (Fages et al, 2000; Hittinen et al, 2000). Huttunen et al. further demonstrated that activation of RAGE by HMGB1 (amphoterin) and S100B can

promote cell survival through increased expression of the anti-apoptotic protein Bcl-2. However, whereas nanomolar concentrations of S100B induced trophic effects in RAGEexpressing cells, micromolar concentrations caused apoptosis in a manner that appeared to depend on oxidant stress. For both of these outcomes, the cytoplasmic domain of RAGE was required, as cells expressing a dominant-negative mutant (i.e., lacking the cytosolic tail) are unresponsive to these stimuli. The neurite outgrowth-promoting role of RAGE was recently confirmed in vivo in a unilateral sciatic nerve crush model, in which blockade of RAGE, either by sRAGE or by blocking F(ab')2 fragments of antibodies (raised to either RAGE or to S100/calgranulins or amphoterin) reduced functional regeneration of the peripheral nerve (Rong et al, 2004a). Similar results were observed in transgenic mice overexpressing dominant negative RAGE (Rong et al, 2004b). However, RAGE−/− mice demonstrate neither obvious neuronal deficits nor overt behavior abnormalities, indicating that RAGE may contribute to neuronal development, but that there are redundant systems that substitute for this receptor in its absence. Furthermore, it will be interesting to see if future experiments in RAGE−/− mice confirm a role for RAGE in the repair of peripheral nerve injury. In terms of a contribution for RAGE in development, expression of the receptor in vivo appears to mirror developmental processes. After being highly expressed during embryonic development, RAGE is downregulated in most organs during normal life (Kokkola et al, 2005). Upon aging, RAGE expression increases again, although it is not known whether this is due to accumulation of RAGE ligands (which upregulate receptor expression) or whether this represents a compensatory mechanism protecting aging cells from cell death. Another line of evidence for a role of RAGE in the regulation of differentiation comes from recently published studies showing that non-small cell lung carcinomas are characterized by downregulation of RAGE (Bartling et al, 2004). One reason for this might be that loss of HMGB1(amphoterin)/RAGE-mediated regulation of tumor cell migration and invasive processes results in more aggressive tumor behavior (Huttunen et al, 2002). A COOH-terminal motif in HMGB1 (amino acids 150–183) has recently been identified as responsible for RAGE binding. This portion of HMGB1 efficiently inhibits RAGE-mediated extension of cellular processes and transendothelial migration of tumor cells. This observation leads us to propose that loss of RAGE might promote tumor growth, at least in settings affecting the lung, one of the few tissues in which RAGE is constitutively expressed at high levels. Since this observation contrasts with a previous finding in which sRAGE suppressed tumor growth and metastasis (Taguchi et al, 2000), the latter observations might be due to the ability of sRAGE to intercept the interaction of RAGE ligands with other receptors.

8. Conclusion and prospective

Cell death from ischemia involves a complex biological cascade. Initially, energy failure is followed by glutamate overload and Ca^{2+} influx into the cell. These processes initiate a series of events, including the generation of free radicals, apoptosis, an inflammatory response and generation of growth factors. Many of these processes are the direct result of the up- or downregulation of specific gene families. Thus, a desirable neuroprotectant would, in theory, be one that antagonises multiple injury mechanisms. The studies described above demonstrate an emerging role for endogenous neuroprotectant in ischemic damage and

ischemic prophylaxis. Among these, erythropoietin (Epo), has a dominant role for neuroprotection, neurogenesis and act as a neurotrophic factor in the central nervous system. These functions make erythropoietin a good candidate for treating disease associated with neuronal cell death. However, our understanding of the underlying mechanisms is far from being complete and a number of open questions remain to be answered: 1) What is the exact route and mechanism through which Epo passes through the BBB? 2) Which cellular mechanisms govern the immunomodulatory effects of Epo in glial cells? 3) Does Epo activate the same or diverse intracellular signaling pathways in the different cells that express EpoR in the brain, neurons, glial, and endothelial cells? Nevertheless, since the discovery of Epo expression in the brain less than 15 years ago, a tremendous achievement in the understanding of its action in the CNS has been accomplished. Today, Epo is a prominent member of a growing list of hematopoietic and angiogenic factors found to be expressed and acting as protective factors in the CNS. Because of the observed increased death rate, rtPA-treated patients should be excluded from acute poststroke EPO application. In cerebral ischemia, albumin is mainly involved in the improvement of blood microcirculation; however, direct neuroprotection cannot be overlooked. Different in vitro and in vivo studies indicate that albumin has direct neuroprotective effects by acting on astrocytes, microglia and neurons. Altogether albumin can alter brain function by many direct and indirect mechanisms and detailed study of these actions will reveal the role of this multifunctional protein in brain functions. Furthermore, the evidence of de novo synthesis of albumin in microglial cells could encourage the neurologist to investigate newer roles of this multifunctional protein in many neurodegenerative diseases. The prothrombotic, proinflammatory state of stroke results in a promotion of thrombin formation and fibrin deposition at the vascular wall, as well as in the formation of platelet-leukocyte coaggregates, leading to severe disturbance of the microcirculation, capillary leakage and tissue damage. The ability of ATIII to inhibit the actions of both factor Xa and thrombin gives it the potential to block, in part or fully, a wide rage of proinflammatory events. Heparin and heparan sulfate glycoproteins (HSPGs) appear to function as receptors for ATIII on endothelium and leukocytes and can lead to the reduced expression of procoagulatory tissue factor and proinflammatory cytokines as well as heterologous receptor regulatory processes. ATIII has been shown in vitro to increase prostacyclin responses and to inhibit a variety of cell responses including endotoxin-induced nuclear translocation of NF-kB, a key step in the generation of the inflammatory response.

Here, we also discussed the critical role of Toll-like receptors in mediating cerebral ischemic injury and suggested endogenous mechanisms that, when induced, redirect this role from detrimental to beneficial. In fact, many diverse neuroprotective paradigms may redirect TLR signaling as one mechanism of endogenous protection. Paradoxically, TLR ligands administered systemically induce a state of tolerance to subsequent ischemic injury. Herein we suggest that stimulation of TLRs prior to ischemia reprograms TLR signaling that occurs following ischemic injury. Such reprogramming leads to suppressed expression of pro-inflammatory molecules and enhanced expression of numerous anti-inflammatory mediators that collectively confer robust neuroprotection. Research findings indicate that numerous preconditioning stimuli lead to TLR activation, an event that occurs prior to ischemia and ultimately leads to TLR reprogramming. Thus genomic reprogramming of

TLR signaling may be a unifying principle of tolerance to cerebral ischemia. Recent studies have also demonstrated an increased expression of the cell-surface RAGE in dying neurons after hypoxic-ischemic insults and human cerebral ischemia, and suggested that the RAGE-ligand interaction causes neuronal cytotoxicity. RAGE also has a circulating truncated variant isoform, soluble RAGE (sRAGE), which corresponds to its extracellular domain only. Exogenously administered sRAGE has been successfully used to antagonize advanced glycation end products (AGE)-RAGE-mediated vascular damage. Accordingly, sRAGE may compete with cell-surface RAGE for the ligand, thus functioning as a decoy and possibly exerting a cytoprotective effect. Most of the data available so far point to the RAGE/NF-κB axis as an attractive target for future clinical interventions in several chronic disease states. However, until physiologic properties of RAGE have been clearly deciphered, it is most prudent to adopt a cautious approach when future therapeutic strategies involving long-term blockade of RAGE or its ligands are considered. Another important issue to be addressed concerns how studies performed in rodent models will translate to human disease. Alternatively, if RAGE antagonists are eventually used in humans, it will be fascinating to understand the impact of long-term blockade of RAGE in critically ill patients, in view of the likely complex role of RAGE, and other receptors interacting with RAGE ligands, in regulating physiologic and pathophysiologic processes in a wide range of situations.

9. References

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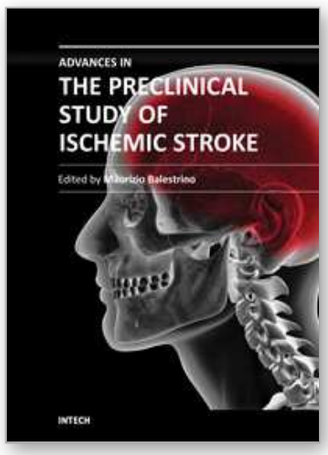

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This book reports innovations in the preclinical study of stroke, including - novel tools and findings in animal models of stroke, - novel biochemical mechanisms through which ischemic damage may be both generated and limited, - novel pathways to neuroprotection. Although hypothermia has been so far the sole "neuroprotection" treatment that has survived the translation from preclinical to clinical studies, progress in both preclinical studies and in the design of clinical trials will hopefully provide more and better treatments for ischemic stroke. This book aims at providing the preclinical scientist with innovative knowledge and tools to investigate novel mechanisms of, and treatments for, ischemic brain damage.

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