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Erythropoietin: Powerful Protection of Ischemic and Post-Ischemic Brain

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Abstract

Ischemic brain injury inflicted by stroke and cardiac arrest ranks among the leading causes of death and long-term disability in the United States. The brain consumes large amounts of metabolic substrates and oxygen to sustain its energy requirements. Consequently, the brain is exquisitely sensitive to interruptions in its blood supply, and suffers irreversible damage after 10–15 minutes of severe ischemia. Effective treatments to protect the brain from stroke and cardiac arrest have proven elusive, due to the complexities of the injury cascades ignited by ischemia and reperfusion. Although recombinant tissue plasminogen activator and therapeutic hypothermia have proven efficacious for stroke and cardiac arrest, respectively, these treatments are constrained by narrow therapeutic windows, potentially detrimental side effects and the limited availability of hypothermia equipment. Mounting evidence demonstrates the cytokine hormone erythropoietin (EPO) to be a powerful neuroprotective agent and a potential adjuvant to established therapies. Classically, EPO originating primarily in the kidneys promotes erythrocyte production by suppressing apoptosis of proerythroid progenitors in bone marrow. However, the brain is capable of producing EPO, and EPO's membrane receptors and signaling components also are expressed in neurons and astrocytes. EPO activates signaling cascades that increase the brain's resistance to ischemia-reperfusion stress by stabilizing mitochondrial membranes, limiting formation of reactive oxygen and nitrogen intermediates, and suppressing pro-inflammatory cytokine production and neutrophil infiltration. Collectively, these mechanisms preserve functional brain tissue and, thus, improve neurocognitive recovery from brain ischemia. This article reviews the mechanisms mediating EPO-induced brain protection, critiques the clinical utility of exogenous EPO to preserve brain threatened by ischemic stroke and cardiac arrest, and discusses the prospects for induction of EPO production within the brain by the intermediary metabolite, pyruvate.

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Introduction

Ischemic syndromes of the central nervous system (CNS) are devastating to the victims and exact an enormous cost on society. Each year nearly 800,000 Americans experience a new or recurrent stroke, of which 87% are ischemic strokes.[1] The fourth leading cause of death and the leading cause of long-term disability in the United States, ischemic stroke kills approximately 130,000 Americans annually,[1,2] and many survivors experience persistent neurocognitive deficits that profoundly impact their quality of life. Nearly 7 million living American adults have suffered a stroke.[2]

Cardiac arrest, *i.e*. sudden cardiac death, which interrupts blood flow to the entire body including the CNS, kills approximately 350,000–400,000 Americans per year, many succumbing to massive brain injury inflicted by the ischemic insult.[3,4] Of the 70,000 cardiac arrest victims initially resuscitated each year in the U.S., approximately 70% of these victims die in the hospital, due primarily to extensive brain damage.[4–6] 40% of initial survivors of cardiac arrest enter a permanent vegetative state, and 80% of them die within 1 year of the event.[7] Only 5–14% of resuscitated victims of cardiac arrest survive without significant cerebral impairment.[8,9] As the American Heart Association's 2008 consensus statement on cardiac arrest laments, "*…little evidence exists to suggest that the in-hospital mortality rate of patients who achieve recovery of spontaneous circulation (ROSC) after cardiac arrest has changed significantly in the past half-century*."[10]

In 2000, White *et al*. commented "*There are as yet no clinically effective therapeutic protocols for amelioration of brain damage by ischemia and reperfusion*."[11] Regrettably this statement still holds true 14 years later. Aside from early restoration of cerebral perfusion, few interventions have been found to prevent ischemic brain injury, despite enormous investments in preclinical and clinical research. Indeed, recombinant tissue plasminogen activator (rtPA) and therapeutic hypothermia are the only interventions with proven clinical efficacy for ischemic stroke and cardiac arrest, respectively. The challenge to any prospective treatment for CNS ischemia is the sheer complexity of the injury cascade triggered by ischemia-reperfusion. This article summarizes research conducted in the last two decades that has demonstrated the natural cytokine erythropoietin to be a potentially powerful neuroprotectant capable of intervening at multiple points in the injury cascade.

Mechanisms of injury in ischemic and post-ischemic brain

Ischemia and reperfusion ignite a complex cascade of brain injury (Figure 1) mediated by glutamate, intracellular Ca^{2+} overload, and reactive oxygen and nitrogen intermediates (RONS). The brain requires continuous delivery of oxygen and energy substrates via the cerebral circulation to sustain its high rate of ATP turnover. Occlusion of cerebral arteries or cardiac arrest interrupts oxidative metabolism, precipitating an abrupt decrease in the

cytosolic Gibbs free energy of ATP hydrolysis (ΔG_{ATP}), the immediate energy source for the ion pumps that manage cytosolic free Ca^{2+} and repolarize the cell membrane. Depolarization of ischemic neurons causes excessive release of the excitatory amino acid neurotransmitter, glutamate.[12–14] Astrocytes normally protect neurons from glutamate toxicity by ATP-dependent sequestration of the neurotransmitter.[15] Loss of ΔG_{ATP} can cause reversal of glutamate transport, so astrocytes release glutamate. Moreover, RONS attack and disable glutamate transporters.

Glutamate binding to α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) and *N*-methyl-*D*-aspartate (NMDA) receptors located on neurons, glia and cerebrovascular endothelium[3] provokes additional depolarization and intense Ca^{2+} entry, sufficient to activate destructive Ca^{2+}-dependent proteases and phospholipases, culminating in cellular injury and death.[11,13,14] Among the Ca^{2+}-activated proteins is calcineurin, which activates the pro-apoptotic protein, Bad, a promoter of mitochondrial permeability transition, and the inducible nitric oxide synthase (NOS) isoform, iNOS, which catalyzes cytotoxic peroxynitrite ($ONOO^-$) formation.[11] Intracellular Ca^{2+} overload also damages neurons by precipitating mitochondrial dysfunction. A spike in cytosolic Ca^{2+} concentration above 0.5 μM increases mitochondrial Ca^{2+} uptake, which provokes sequential opening of the mitochondrial permeability transition pores, collapse of the inner mitochondrial membrane potential, failure of oxidative phosphorylation, and generation of RONS.[14]

By binding to NMDA receptors, glutamate activates NOS[16,17] to produce excessive amounts of NO which condense with superoxide ($\bullet O_2^-$), yielding a cytotoxic product, $ONOO^-$.[18] At the onset of reperfusion there is a burst of RONS formation in the brain,[19] with microglia as a major source of NO.[20,21] In addition, ischemia-reperfusion can induce iNOS in astrocytes, causing these cells to release toxic amounts of NO. $ONOO^-$ initiates peroxidation of membrane phospholipids, nitrosylates tyrosine and cysteine residues in proteins, and depletes the intracellular antioxidant, glutathione.[18,22] Moreover, $\bullet O_2^-$ reacts with heme, liberating Fe^{2+} which catalyzes lipid peroxidation.[11] Hypothermic circulatory arrest in dogs activated cerebrocortical neuronal NOS (nNOS), which peaked at five times the pre-ischemic activity at 20 h post-arrest.[23] In a rat model of *status epilepticus*, bilateral microinjection of kainate induced hippocampal NO, $\bullet O_2^-$ and $ONOO^-$ formation, which led, sequentially, to inactivation of mitochondrial respiratory complex I, cytochrome *c* release, initiation and propagation of caspase activity and, finally, DNA fragmentation.[24]

Calcium[25] and RONS[26,27] induce astrocytes,[25,26,28] microglia[25] and cerebrovascular endothelium[29–31] to secrete matrix metalloproteinases (MMPs), a class of enzymes that degrade protein components of the extracellular matrix and of the tight junctions within the capillary endothelium that comprise the blood-brain barrier (BBB).[32–35] By oxidizing cysteine residues in the autoinhibitory domain of proMMPs, RONS activate MMPs by the 'cysteine switch' mechanism.[36] MMPs have been implicated in BBB disruption and brain edema and inflammation.[37,38] Interstitial brain edema, which develops within 1 hour after cardiac arrest or stroke[3] is associated with poor neurological outcome. Brain edema increases intracranial pressure, which compresses the brain, lowers cerebral perfusion pressure and decreases cerebral blood flow. Moreover, BBB disruption allows neutrophils to infiltrate the brain parenchyma, where they release RONS and MMPs that further

compromise the BBB. In rats subjected to cardiac arrest – CPR, neutrophils were detected in the susceptible brain regions within 6 h ROSC.[9]

Neuronal apoptosis after brain ischemia and reperfusion

Brain ischemia triggers two general processes of neuronal death: necrosis and apoptosis.[39,40] Which process predominates depends on the duration and intensity of the ischemic insult. In focal ischemia, necrosis is the major cause of cell death in the intensely ischemic core.[41] The core is surrounded by the less severely ischemic penumbra, where neurons primarily die by apoptosis, a highly regulated mechanism of cell death.[39,40,42,43] Because apoptosis is orchestrated by specific signaling elements, and because its measured pace affords time to initiate treatment, there are opportunities to salvage penumbral cells threatened by ischemic stroke.

Two distinct apoptotic cascades operate in the CNS (Figure 2).[39,40,44] In the extrinsic pathway, Fas ligand secreted by neurons, glia and inflammatory leukocytes binds its receptor, Fas, which, via its Fas-activated death domain, activates caspase 8, a protease that mediates apoptosis by activating caspase 3, the major 'executioner' caspase, and cleaves Bid to truncated Bid (*t*Bid), which combines with Bad in the mitochondrial membrane forming a channel. The release of cytochrome *c* through this channel initiates the intrinsic apoptotic pathway. In the cytosol, cytochrome *c* combines with Apaf-1, dATP and procaspase 9, forming the apoptosome which activates caspase 9 by cleavage of its procaspase. In a similar manner, caspase 9 activates caspase 3, which cleaves numerous targets culminating in the cell's destruction.

Neuronal apoptosis is well documented in animal models of cardiac arrest. For example, in rabbits placed on cardiopulmonary bypass and subjected to 2 h hypothermic circulatory arrest, 4 h reperfusion, hippocampal CA1 neurons exhibited caspase-3 activation and DNA fragmentation detectable by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL).[45] Böttiger, Teschendorf *et al*.[46,47] examined the progression of apoptotic cell death in rat brain over the first 7 d recovery from cardiac arrest – CPR. Post-arrest caspase activity followed different time-courses in different brain regions. In *nucleus reticularis thalami*, cortex and striatum, caspase activity and DNA fragmentation detected by TUNEL were already maximal at 6 h ROSC. In the hippocampal CA1 subregion, TUNEL-positive cells were first detected at 3 d, and increased further at 7 d. Thus, cardiac arrest activates caspases and apoptosis in vulnerable brain regions. A strong correlation emerged, both in extent and time-course, between caspase activation and DNA fragmentation.

Nitric oxide generated by the neuronal and inducible NOS isoforms has been implicated in CNS apoptosis following cardiac arrest. Incubation of hippocampal neurons with the NO donor sodium nitroprusside lowered Bcl-2 content and increased Bax content, and activated caspase-3.[48] In astrocyte-neuron cocultures, NOS inhibition by *L*-NMMA increased neuronal survival and prevented the decrease in Bcl-2 and increase in Bax initiated by hypoxia-reoxygenation.[49]

Erythropoietin: cerebroprotective cytokine

Erythropoietin, a 165 amino acid, 30.4 kDa glycoprotein with four oligosaccharide chains, was identified over 30 years ago as the hormone responsible for inducing erythropoiesis.[50] The liver is the major source of EPO during the prenatal period. Postpartum, 90% of EPO production shifts to the kidneys,[51] where peritubular interstitial fibroblasts near the corticomedullary border synthesize and secrete EPO in response to hypoxemia.[52–54] EPO circulates to the bone marrow, where it suppresses apoptosis of colony-forming unit erythroid cells, promoting the proliferation and development of these cells into mature erythrocytes.[50,55] EPO's anti-apoptotic protection of erythroid precursors was an early indication that the cytokine might similarly protect cells in other tissues, including brain.

Studies in a variety of animal models of CNS ischemia-reperfusion[56,57] have defined EPO's robust neuroprotective properties in brain.[58–61] In stroke-prone spontaneously hypertensive rats, cerebroventricular infusion of EPO salvaged cerebral cortex and motor function following permanent middle cerebral artery (MCA) occlusion.[62] The abundance of mRNA encoding the EPO receptor was elevated in the ischemic penumbra, potentially enhancing the neuroprotective capabilities of EPO and preventing infarct expansion. Injection of EPO (5,000 IU/kg, *ip*) at the start of 60 min MCA occlusion in rats decreased infarct size by 75% and suppressed apoptosis in the ischemic penumbra.[63] Erythropoietin (1,000 IU/kg, *ip*) decreased ethanol-induced apoptosis in cerebellum, prefrontal cortex, and hippocampus of mice given subcutaneous ethanol injections.[64] In gerbils subjected to 5 min bilateral carotid artery occlusion,[65] recombinant human EPO, when injected (50 or 100 IU, *ip*) at the time of reperfusion, attenuated hippocampal edema, lipid peroxidation and neuronal death, and suppressed NO formation. Thus, EPO treatment may protect sensitive brain regions, at least in part by suppressing NOS.

Transgenic human EPO expression in mouse brain doubled cerebrocortical and striatal EPO content *vs*. wild type, and decreased infarct volume by 84% following 90 min middle cerebral artery occlusion and 72 h reperfusion.[66] In this study, TUNEL-positive and caspase-3-positive neurons were decreased by ∼50 and ∼75%, respectively, in transgenic *vs*. wild-type striatum. EPO expression sharply increased phosphor-activation of Erk-1, Erk-2 and Akt; the Erk inhibitor PD98059 and the PI3K/Akt inhibitor Wortmannin both prevented the reduction in TUNEL- and caspase-3-positive neurons, implicating both kinases in the neuroprotective cascade.

EPO has been found to be cerebroprotective even when its administration is delayed. In rats, exogenous EPO decreased infarct volume even when given 6 h after MCA occlusion-reperfusion.[67] In a rat model of traumatic brain injury, EPO (5,000 IU/kg, ip) given 24 h post-injury produced significant improvement in neurological function and decreased neuronal loss in the hippocampal CA3 subregion, and increased neurogenesis in the injured cortex and dentate gyrus.[68] Erythropoietin, injected *ip* in rats subjected to MCA occlusion, reduced infarct volume by 70–75% whether given 24 h before, during or 3 h after occlusion.[63] EPO also sharply lowered TUNEL-positive cells in the ischemic penumbra of these rats. Importantly, some protection was still seen when EPO was administered as late as 6 h post-occlusion, although not at 9 h post-occlusion. EPO's neuroprotective efficacy for at

least the first several h after the ischemic insult expands opportunities for its therapeutic application for acute CNS ischemia.

Although the preponderance of preclinical evidence shows EPO to be neuroprotective, a study in rats subjected to 6 min pre-treatment ventricular fibrillation, 2 min CPR, defibrillatory countershocks and up to 7 d recovery yielded less favorable outcomes.[69] EPO (5000 IU/kg), given *iv* 5 min before cardiac arrest, then injected *ip* at 24 and 72 h post-arrest, failed to suppress total caspase or caspase-3 activities, prevent DNA fragmentation and neuronal degeneration in the hippocampal CA1 subregion, or improve neurological deficit score at 1, 3 or 7 d recovery. These negative findings merit attention in light of the equivocal results of clinical trials of EPO for CNS ischemia described below.

Mechanisms of erythropoietin neuroprotection

Erythropoietin is an especially promising neuroprotectant because it potentially intervenes at several points in the apoptotic pathway (Figure 2). Brain neurons express homodimeric EPO receptors; EPO binding triggers reciprocal auto-phosphorylation of the two monomers, which in turn phosphorylate and activate the signaling kinase, Jak-2.[70] Multiple protein kinases are recruited to the EPO receptor and phosphorylated by activated Jak2, initiating a complex antiapoptotic signaling cascade (Figure 2). Several cytoprotective mechanisms activated by EPO signaling are summarized in the following subsections.

Increased anti-apoptotic proteins and Bcl-X_L/Bax ratio

The relative cellular contents of anti- *vs*. pro-apoptotic members of the Bcl protein family exert a profound effect on cell survival *vs*. apoptosis.[71,72] EPO enhancement of neuronal Bcl-X_L content plays a pivotal role in EPO's anti-apoptotic neuroprotection.[60] In cultured rat cortical microglia and astrocytes, EPO shifted the Bcl/Bax ratio in favor of anti-apoptotic Bcl.[73] In gerbils subjected to CNS ischemia, EPO up-regulated Bcl-X_L mRNA and protein in hippocampal CA1 neurons, and prevented learning disability.[74] Transgenic over-expression of human EPO in murine striatum enhanced ischemic induction of Bcl-X_L.[66] Activated Akt phosphorylates the proapoptotic protein, Bad, preventing the latter's insertion into the mitochondrial membrane.[75] Phosphorylated STAT5 activates nuclear factor κB (NF-κB), which promotes expression of the anti-apoptotic proteins X-linked inhibitor of apoptosis (XIAP) and c-inhibitor of apoptosis-2 (cIAP2) in cultured cerebrocortical neurons.[76] c-IAP2 suppresses caspases 3, 8 and 9;[77] XIAP binds and suppresses caspases 3 and 9,[78] and inhibits activation of procaspase 9 within the apoptosome.[79]

Enhancement of the brain's antioxidant defenses

Preclinical studies have demonstrated EPO induction of key components of the brain's antioxidant armamentarium. In rats, *ip* injection of 1,000 IU/kg EPO at 8 h intervals beginning 5 min after induction of subarachnoid hemorrhage increased gene expression and content of the antioxidant enzymes glutathione *S*-transferase, NAD(P)H:quinone oxidoreductase-1 and heme oxygenase-1, and blunted cerebrocortical apoptosis, brain edema and BBB disruption 48 h later.[80] EPO (1,000 IU/kg, *ip*) increased glutathione peroxidase activity and decreased lipid peroxidation in the brains of ethanol-intoxicated mice.[64] In

brains of rats subjected to hyperoxia-imposed oxidative stress, EPO (20,000 IU/kg, *ip*) upregulated heme oxygenase-1, dampened lipid peroxidation, and prevented the decline in glutathione redox state.[81]

Recent studies implicate the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) in EPO's induction of antioxidant enzymes. Nrf2 activates expression of a gene program encoding several phase II defense enzymes that afford antioxidant and anti-inflammatory cytoprotection,[82,83] including heme oxygenase-1, peroxiredoxin, superoxide dismutase, glutathione peroxidase, NAD(P)H:quinone oxidoreductase-1, and the glutathione synthesizing enzyme glutamate-cysteine ligase.[80,84,85] Binding of a regulatory protein, Keap1, sequesters Nrf2 in the cytoplasm, targeting Nrf2 for polyubiquitinylation and proteasomal degration and, thus, silencing the Nrf2 gene program.[86–88] RONS oxidize Keap1 sulfhydryls,[83] liberating Nrf2 which translocates to the nucleus and binds the antioxidant response element in the promoter of phase II response genes. EPO is proposed[89] to activate Nrf2 by activating Akt and Erk, which in turn phosphor-activate eNOS, thereby increasing NO formation in the neuronal cytosol (Figure 2). NO or its derivative $ONOO^-$ release Nrf2 by nitrosylating Keap1's regulatory sulfhydryls.[90] Accordingly, pharmacological inhibition of Akt and Erk blunted EPO-induced nuclear translocation of Nrf2 and heme oxygenase-1 expression in cultured human neural cells.[84]

Suppression of matrix metalloproteinases and inflammation

Li *et al*.[91] studied mice subjected to intracerebral hemorrhage, a pro-inflammatory event. EPO (*ip* injection), given during the first 3 d post-hemorrhage, preserved the BBB, prevented tissue edema, preserved collagen, restrained increases in MMP-2 content and enhanced content of the endogenous MMP inhibitor, tissue inhibitor of metalloproteinase-2 (TIMP-2). In human erythroid progenitor cells, EPO suppressed MMP-9 secretion and induced TIMP-1 expression and secretion.[92] ERK1/2 inhibitors PD98059 and U0126 and PI3K inhibitor LY294002 blocked EPO suppression of MMP-9 and induction of TIMP-1. These findings are empirical evidence that EPO preserves the extracellular matrix and prevents CNS injury by inducing TIMPs and suppressing MMPs. In rats undergoing MCA occlusion, EPO (5000 IU/kg body wt, *ip*) decreased astrocyte activation and recruitment of leukocytes and microglia into the infarct, and suppressed formation of the pro-inflammatory cytokines IL-6, TNF and monocyte chemoattractant protein-1 by >50%.[93]

Erythropoietin dampens glutamate excitotoxicity

The excitatory amino acid glutamate provokes neuronal Ca^{2+} entry via NMDA and AMPA channels. Excessive glutamatergic activity in ischemic and post-ischemic brain provokes cytotoxic Ca^{2+} overload. EPO suppressed glutamate release from hippocampal and cerebellar neurons exposed to 'chemical ischemia' produced by excess Ca^{2+} or ionomycin,[94] in spinal neurons exposed to excitotoxic kainic acid[95] and in electrically stimulated hippocampal slices.[96] By dampening glutamate release, EPO may ameliorate NMDA- and AMPA-channel-mediated Ca^{2+} entry, thereby preventing excitoxicity and minimizing ATP demands for Ca^{2+} extrusion by the energy-depleted neurons.

Erythropoietin modulation of nitric oxide synthase

Erythropoietin exerts divergent effects on the three NOS isoforms. EPO dampened expression of iNOS in oligodendrocytes exposed to inflammatory stimuli.[89] Transgenic expression of human EPO in murine brain suppressed nNOS and iNOS expression in striatal neurons.[66] In gerbils subjected to bilateral carotid occlusion, post-ischemic EPO injection (*c*. 800–1500 100 IU/kg, *ip*) 60 min after reperfusion lowered NO formation in the hippocampus, in parallel with EPO's suppression of lipid peroxidation and tissue edema.[65] Neuronal NOS is Ca^{2+}-activated, so EPO's suppression of glutamatergic signaling and the resultant Ca^{2+} overload may contribute to the decreased NOS activity. In contrast, EPO has been shown to activate the endothelial NOS isoform (eNOS), which generates the moderate amounts of NO which activate Nrf2.[84,89,90]

Clinical trials: exogenous erythropoietin for brain ischemia

As Pytte and Steen[97] noted, "*…the last three decades have been filled with disappointments regarding pharmacological treatment of cardiac arrest patients*." Indeed, an array of potential treatments has failed to impart significant clinical benefit, including treatments which afforded substantial neuroprotection in animal models. Clinical trials of EPO for brain ischemia have yielded mixed outcomes. Ehrenreich *et al*.[98] conducted a pioneering clinical trial in which *iv* injections of 33,000 IU EPO, daily for the first 3 days after stroke, improved recovery of neurocognitive function and decreased the persistent neurological deficit evident 18–30 d after stroke. EPO was efficacious when the first dose was given up to 8 h after the onset of stroke symptoms, but massive doses of EPO were required for clinical benefit.

Cariou *et al*.[99] conducted a clinical trial of EPO for brain protection following cardiac arrest. Five intravenous injections of 40,000 IU EPO at 12 h intervals, beginning 42–72 min after out-of-hospital cardiac arrest, failed to improve neurological recovery assessed at day 28 post-arrest. EPO did produce modest increases in hematocrit and hemoglobin content at 14 d post-arrest *vs*. non-EPO controls. A small trial by Grmec *et al*.[100] showed that a single, massive *iv* bolus of EPO (90,000 IU), given by emergency responders within 1–2 min of initiating CPR, did increase rates of initial defibrillation, survival to ICU admission, 24 h survival and survival to hospital discharge. Despite these promising short-term outcomes, EPO treatment did not improve neurological outcome.

Ehrenreich *et al*.[101] studied 460 patients with stroke in the MCA perfusion territory. Patients received three *iv* injections of 40,000 IU EPO, at 6, 24 and 48 h after onset of symptoms. EPO increased death rate (16.4%; 42/256) *vs*. placebo (9.0%; 24/266) and incidence of cerebrovascular hemorrhage. These adverse effects were seen almost entirely in patients receiving recombinant tissue plasminogen activator (rtPA) beyond its therapeutic window, which is limited to the first 4.5 h after stroke onset.[102,103]

A recent preclinical study by Jia *et al*.[104] provided valuable insights regarding the detrimental interaction of rtPA and EPO. Rats were subjected to embolic MCA occlusion, followed by EPO (5000 IU/kg, *ip* injection) and rtPA treatment (10 mg/kg, *iv* injection) at 2 or 6 h MCA occlusion. When administered at 2 h MCA occlusion, EPO and rtPA were similarly effective at reducing infarct size, but the combination of the two afforded no

additional protection over the separate treatments. When administered at 6 h MCA occlusion, although EPO alone decreased infarct size, neither rtPA alone or combined with EPO afforded protection. Indeed, rtPA increased intracerebral hemorrhage at 6 h MCA occlusion *vs*. saline-injected control rats, and the combined EPO + rtPA treatment increased intracerebral hemorrhage even more than rtPA alone. The combined treatments, but not EPO or rtPA alone, activated MMP-9 via nuclear factor κB (NF-κB) signaling in cerebral microvessels at 6 h MCA occlusion. Thus, when EPO and rtPA are coadministered beyond rtPA's therapeutic window, the result is activation of MMP-9, culminating in cerebral hemorrhage and infarct expansion.

How readily does erythropoietin traverse the blood-brain barrier?

The transfer of systemically administered EPO from the cerebral circulation across the BBB into the brain parenchyma is less than 1% efficient;[67,105,106] consequently, high doses are required to achieve therapeutically effective EPO concentrations within the brain.[60] In mice a tiny fraction of intravenously injected EPO, 0.05–0.1% of the injected dose, entered the brain parenchyma, an efficiency that approximated that of albumin.[105] In fetal sheep and monkeys injected with high doses of EPO, the EPO activity in the cerebrospinal fluid was only about 2% of the circulating activity.[106] Similar results were reported in humans;[107] indeed, the dosages of recombinant EPO required to produce neuroprotection (1,000–30,000 IU/kg) are well above those (<500 IU/kg) used to treat anemia.[108] Other studies showed that circulating EPO can only enter the brain if the BBB has been compromised. In patients with traumatic brain injury, the appearance of EPO in the ventricular cerebrospinal fluid correlated with the extent of BBB disruption.[109] In a patient undergoing resection of a brain tumor, a single *iv* injection of 6000 IU recombinant human EPO increased serum EPO activity from *c*. 13 to >6500 IU/l for at least 60 min, but there was no increase in EPO activity in the cerebrospinal fluid.[110] Collectively, these studies demonstrate that circulating EPO does not efficiently cross the intact BBB, but can pass from blood to brain if the BBB is disrupted. The high doses of exogenous EPO necessary to surmount the intact BBB may increase blood coagulability enough to precipitate thrombotic events[111] and, when combined with tPA therapy, produce deadly hemorrhagic transformation.[104,112]

Erythropoietin expression within the brain

Noguchi *et al*.[75] stated "*EPO production in neural cells can increase the local bioavailability of EPO independent of transit through the blood-brain barrier*." The brain possesses the molecular machinery to manufacture EPO intrinsically, on the "leeward" side of the blood-brain barrier.[59,113–115] Indeed, EPO mRNA abundance in the cerebellum, pituitary gland and cerebrocortex rivaled that of the conventionally EPO-expressing liver and kidneys.[116] Substantial EPO expression was detected in several brain regions[116] and spinal cord[117] in preterm human fetuses. Nagai *et al*.[118] examined expression of EPO and its receptors in cultured human astrocytes, neurons, microglia and oligodendrocytes. Only the astrocytes expressed EPO mRNA. Neurons, astrocytes and microglia possessed EPO receptors; the oligodendrocytes did not. In gerbils, sequestration of intrinsic EPO by injection of soluble EPO receptors into the cerebral ventricles intensified neuronal death in the hippocampus following a moderate, ordinarily non-injurious ischemic challenge,[119]

suggesting that EPO production within the brain contributed to a basal level of neuroprotection.

As in kidney,[120,121] hypoxia is a powerful inducer of EPO expression in brain.[94,122] This induction is mediated by hypoxia inducible factor-1 (HIF-1), an O_2-regulated transcription factor that activates the expression of an extensive gene program encoding proteins that increase cellular resistance to hypoxia and ischemia.[51,123] HIF-1 is a heterodimer containing two subunits: a constitutive β subunit and an α subunit which is also constitutively expressed but, in well-oxygenated tissues, rapidly undergoes prolyl hydroxylase-catalyzed, Fe^{2+}- and α-ketoglutarate-dependent hydroxylation of two prolyl residues, earmarking the subunit for poly-ubiquitinylation and proteosomal degradation (Figure 2).[124] Hypoxia stabilizes HIF-1α in two ways:[114] it deprives prolyl hydroxylase of the O_2 required for HIF-1α hydroxylation, and it causes the mitochondrial electron transport chain to generate RONS which convert Fe^{2+} to Fe^{3+}, removing the source of electrons for the prolyl hydroxylase reaction. Thus stabilized, HIF-1α diffuses from the cytosol to the nucleus and combines with the β subunit, forming the active HIF-1 transcription factor. HIF-1 then binds the hypoxia response element in the promoter regions of an extensive array of genes, including EPO, vascular endothelial growth factor, the entire glycolytic enzyme sequence, and a host of other proteins which, collectively, increase cellular resistance to hypoxia and ischemia.[114] Thus, embryonic mouse neocortical neurons and astrocytes expressed EPO mRNA and protein when exposed to hypoxia or the hypoxia-mimetic chemicals desferrioxamine or cobalt chloride.[125] While EPO is intensely expressed by astrocytes, its membrane receptors are predominantly located in neurons and cerebrovascular endothelium. EPO secreted by astrocytes may function in a paracrine manner (Figure 2).

By effectively surmounting the BBB, while potentially avoiding the untoward effects of massive systemic EPO dosages, intrinsic EPO expression within the brain parenchyma addresses the important limitations of exogenous EPO. However, a strategy of subjecting critically ill patients to systemic hypoxia in the midst of an acute CNS ischemic event would be dangerous and clinically unacceptable. Is there a safe, simple means of inducing EPO expression in the brain for treatment of acute CNS ischemia?

Neuroprotection by exogenous pyruvate

The neuroprotective capabilities of pyruvate, a natural intermediary metabolite and energy substrate, have been demonstrated in a variety of brain preparations. Although an exhaustive review of these studies is beyond the scope of this article, several reports exemplifying the neuroprotection afforded by pyruvate are summarized here. Lee *et al.*[126] subjected rats to 12 min forebrain ischemia by bilateral occlusion of the carotid arteries. Sodium pyruvate (250, 500 or 1000 mg/kg) sharply lowered mortality to 1 of 26 rats *vs.* 18 of 31 NaCl-injected control rats when injected *ip* at 30 min or 1 h reperfusion, but was ineffective when given at 2 or 3 h reperfusion. In the NaCl-injected rats, extensive cell death was detected in the post-ischemic brain 72 h after ischemia-reperfusion; pyruvate (500 mg/kg) prevented cell death. Thus, pyruvate injected *ip* protected brain from ischemia, even when given 30 or 60 min after reperfusion. In a swine model of hemorrhagic shock, Mongan *et al.*[127] showed that intravenous resuscitation with sodium pyruvate suppressed excitotoxic glutamate release

within the cerebral cortex and slowed the post-hemorrhage decline in cortical electrical activity. Kim *et al*.[128] studied kainate-induced epileptic seizures in rats. Sodium pyruvate (500 mg/kg, *ip*) was injected 30 or 150 min after kainate (10 mg/kg, *ip*). Pyruvate sharply lowered, by 60–85%, cell death in hippocampal CA1, CA3 and dentate gyrus. Zinc injures neurons by activating metallothioneins, interfering with mitochondrial respiration, inducing ROS formation by the respiratory chain, and activating NADPH oxidase to produce $\cdot O_2^-$. Pyruvate prevented intracellular zinc accumulation in the studies of Lee *et al*.[126] and Kim *et al*.[128]

In a study by Sharma *et al*.,[129] pyruvate prevented simulated ischemia-induced damage and death of cultured rat astrocytes subjected to simulated ischemia-reperfusion. Cells were challenged by 6 h profound, substrate-free hypoxia, then reoxygenated for another 6 h in presence of pyruvate or glucose. Pyruvate maintained cellular morphology, prevented lactate dehydrogenase leakage, a measure of membrane rupture and cell death, and suppressed early apoptotic events including mitochondrial cytochrome *c* release, caspase-3 cleavage and activation, and poly(ADP-ribose) polymerase (PARP) cleavage, in a manner superior to glucose.

In anesthetized dogs, Sharma *et al*.[130] evaluated pyruvate protection of the brain threatened by cardiac arrest and resuscitation. The heart was arrested by epicardial shock, then, after 5 min arrest, cardiac massage was performed for 5 min before defibrillation by epicardial countershocks. Sodium pyruvate or NaCl were infused *iv* ($0.125\ mmol \cdot kg^{-1} \cdot min^{-1}$) during cardiac massage and the first 60 min recovery, and then the dogs were recovered for 3 days. The pyruvate infusion increased arterial plasma pyruvate concentration from 0.22 ± 0.02 to 3.6 ± 0.2 mM; pyruvate concentration subsided within 30 min post-infusion.[131] Pyruvate sharply lowered neurological deficit 24 and 48 h post-arrest, particularly the deficits in motor function, *vs*. the NaCl-infused dogs. Pyruvate also lowered neuronal death and caspase-3 activity in the hippocampal CA1 subregion and prevented degeneration of cerebellar Purkinje cells.

Fukushima *et al*.[132] demonstrated pyruvate protection of brain in a rat model of cortical contusion injury. Sodium pyruvate was injected (500 or 1000 mg/kg, *ip*) 5 min after contusion. Intracerebral pyruvate detected by microdialysis plateaued at 30–75 min after pyruvate injection, confirming that pyruvate traversed the BBB in this model. Both doses of pyruvate sharply lowered the intensity of cortical cell death at 6 h post-contusion.

Recently, Ryou *et al*.[133] examined pyruvate's neuroprotective capabilities in a rat model of ischemic stroke, in which the left MCA was occluded by advancing a suture into the artery for 120 min; suture withdrawal abruptly reperfused the ischemic tissue. Sodium pyruvate or NaCl control were infused *iv* from 60 min occlusion until 30 min reperfusion. Analyses of brains harvested at 24 h reperfusion revealed that pyruvate infusion produced an 84% reduction in infarct volume and 80% reduction in apoptotic nuclei *vs*. the respective control values. Indeed, the reduction in infarct volume afforded by pyruvate was nearly identical to that produced by transgenic human EPO expression in Kilic *et al*.'s studies in mice subjected to MCA occlusion-reperfusion.[66] Collectively, these and other reports

demonstrate that timely administration of pyruvate can minimize brain injury from ischemia-reperfusion and other stresses.

Pyruvate traverses the blood brain barrier

Many potentially cerebroprotective compounds have proven ineffective due to their inability to surmount the BBB. In contrast, pyruvate is readily transferred across the BBB by a high-affinity, proton-linked monocarboxylate transport mechanism in the vascular endothelium (Figure 3).[134,135] Monocarboxylate transporters also are abundant in the plasma membranes of neurons and astrocytes,[136] affording pyruvate uptake by the brain parenchyma. Using cerebrocortical microdialysis in a pig model of hemorrhagic shock, Mongan *et al*.[127] showed that intravenous pyruvate (0.9 mmol • kg^{-1} bolus followed by 0.08 mmol • kg^{-1} • min^{-1} infusion), producing a sustained arterial plasma pyruvate concentration of 5–6 mM, increased pyruvate concentration in cerebrocortical microdialysate from 0.09 to 0.43 mM. Although the fractional recovery of pyruvate in the microdialysate wasn't reported, the results suggest pyruvate does indeed cross the blood-brain barrier, but doesn't equilibrate. On the other hand, the neurons and astroglia may have avidly taken up the pyruvate, keeping the interstitial concentration low. Cerebrocortical microdialysis studies in rats by Fukushima *et al*.[132] confirmed that pyruvate, injected *ip* appeared in the brain parenchyma over a period of several minutes. Additional evidence that pyruvate cerebroprotection requires pyruvate transport was reported by Wang *et al*.,[137] who showed *ip* injections of 500 mg/kg sodium pyruvate decreased infarct size nearly 50% in rats subjected to 65 min MCA occlusion, and that this cerebroprotective effect was blunted by the monocarboxylate transporter antagonist α-cyano-4-hydroxycinnamate.

Cerebroprotective mechanisms of pyruvate

Pyruvate may preserve post-ischemic brain by several mechanisms. An energy-yielding, oxidizable fuel,[138,139] pyruvate augments oxidative metabolism, thereby generating ATP and phosphocreatine[127] and, thus, increasing ΔG_{ATP}, the thermodynamic driving force for cellular function. Pyruvate also affords three general antioxidant mechanisms:[139,144] (1) as an alpha-keto carboxylate, pyruvate can react with and directly detoxify H_2O_2, lipid peroxides and $ONOO^-$;[140–142] (2) pyruvate oxidizes the cytosolic NADH/NAD^+ redox couple, thereby decreasing availability of NADH to NADH oxidase, which generates $\bullet O_2^-$;[143] (3) pyruvate bolsters intracellular antioxidant defenses by increasing NADPH/$NADP^+$ and, thus, glutathione redox state, the major intracellular antioxidant system.[131,145] Pyruvate suppressed DNA fragmentation, a critical event in the progression of apoptosis (Figure 2) in a cultured renal tubular epithelial cell line subjected to antimycin A-induced chemical hypoxia,[146] as well as in H_2O_2-challenged mouse thymocytes[147] and post-ischemic rat liver.[148] Pyruvate suppression of H_2O_2-induced glutathione depletion, caspase activation and death of cultured human umbilical vein endothelial cells[149,150] paralleled intense Erk1/2 phosphorylation[150] as well as increased Bcl-2 and decreased Bax contents and, thus, increased anti-apoptotic Bcl-2/Bax ratio.[149] Although pyruvate's actions in cerebrovascular endothelium are not yet known, effects such as these could stabilize integrity of the cerebrovascular endothelium and blood brain barrier in the face of ischemia-reperfusion.

Several reports over the past decade have demonstrated pyruvate's antioxidant and anti-apoptotic actions in brain preparations. Wang *et al*.[151] showed that cultured astrocytes released pyruvate which protected co-cultured neurons from copper-catalyzed cysteine autoxidation, a source of hydroxyl radicals. In rat primary neurons, 2.5 mM pyruvate suppressed β-amyloid-induced dichlorofluorescein fluorescence, a measure of ROS formation.[152] In another study[153] pyruvate protected murine neuroblastoma cells from cell death triggered by H_2O_2 and 6-hydroxydopamine, an inducer of H_2O_2 formation. Wang *et al*.[154] exposed cultured human neuroblastoma SK-N-SH cells to 150 μM H_2O_2, which provoked mitochondrial superoxide formation, collapsed the mitochondrial membrane potential, and killed 85% of the cells. Pyruvate concentration-dependently suppressed cell death; 1–4 mM pyruvate completely prevented H_2O_2-induced cell death, even when its administration was delayed until 1 h after H_2O_2 exposure. Pyruvate also suppressed H_2O_2-induced intracellular and mitochondrial RONS formation, with 2 mM pyruvate exerting near-complete prevention of RONS. Massive mitochondrial depolarization by 3 mM H_2O_2 was prevented by 1 mM pyruvate.

Pyruvate's anti-inflammatory actions have been demonstrated in several organs, including brain. Cardiopulmonary bypass provokes a systemic inflammatory response that damages internal organs and compromises post-surgical recovery.[155,156] In pigs subjected to cardioplegia-induced cardiac arrest and maintained on-pump, pyruvate-fortified cardioplegia suppressed the pro-inflammatory C-reactive protein, enhanced anti-inflammatory cytokine IL-10, prevented activation of MMP-9, suppressed neutrophil infiltration into the myocardial parenchyma, and blunted nitrotyrosine formation, a measure of nitrosative stress.[157] These effects were seen 4 h after pyruvate treatment. In dogs, cardiac arrest and cardiopulmonary resuscitation produced a striking increase in hippocampal MMP activity 3 d later; pyruvate infusion during cardiac massage and the first 60 min recovery suppressed this MMP activation by 80%.[130] Sharma and Mongan[158] examined the anti-inflammatory capabilities of low-volume, hypertonic sodium pyruvate resuscitation in a rat model of hemorrhagic shock. The pyruvate treatment ameliorated liver injury, suppressed serum and hepatic pro-inflammatory cytokines, NOS and cyclooxygenase-2 activities, caspase-3 activation and poly(ADP ribose) polymerase cleavage and lipid peroxidation, and attenuated liver injury. Thus, pyruvate can supply energy substrate, detoxify RONS and suppress inflammation and apoptosis in CNS threatened by acute ischemia-reperfusion.

Induction of erythropoietin and neuroprotection by pyruvate

Studies in a cultured human glioma cell line revealed a novel action of pyruvate: the stabilization of HIF-1α despite the presence of abundant O_2.[159,160] Here, pyruvate and oxaloacetate, an α-keto carboxylate structural analogue and product of mitochondrial pyruvate carboxylation (Figure 3),[139] suppressed prolyl hydroxylase activity, apparently by competing with the enzyme's natural substrate, α-ketoglutarate, for access to the enzyme's catalytic domain.[161] These findings raised the possibility that pyruvate could suppress prolyl hydroxylation and subsequent polyubiquitination and degradation of HIF-1α and, thus, augment expression of HIF-1-activated genes, including EPO, in normal tissue.

Ryou *et al*.'s studies in a porcine cardiopulmonary bypass model revealed, for the first time, pyruvate induction of EPO synthesis in a mammalian organ, the heart.[162] Here, pyruvate-enriched cardioplegia stabilized HIF-1α content, which paralleled robust myocardial mRNA expression and synthesis of EPO. Elements of EPO's intracellular signaling cascades, Erk and eNOS, were activated following pyruvate cardioplegia. Thus, temporary (60 min) pyruvate treatment evoked EPO expression and its cytoprotective signaling cascades that persisted several h after treatment. Indeed, the myocardium released EPO into the coronary venous effluent for at least 4 h after crossclamp release and washout of the pyruvate-enriched cardioplegia.

In Ryou *et al*.'s rat model of ischemic stroke,[133] pyruvate treatment increased cerebral EPO content severalfold, in the ischemic tissue as well as the contralateral, non-ischemic hemisphere. Additional experiments were conducted in glioma and neuronal cell lines subjected to oxygen-glucose deprivation and reoxygenation, a cell culture model of ischemia-reperfusion, to assess the roles of HIF-1α, EPO and the downstream signaling in pyruvate's neuroprotection.[133] Five and 10 mM pyruvate afforded significant cytoprotection, paralleled by marked increases in HIF-1α and EPO contents and phosphor-activation of Akt but not Erk. Incubation with soluble EPO receptor, and siRNA suppression of HIF-1α expression, blunted pyruvate's cytoprotection. Collectively, these results support the hypothesis that pyruvate prevents ischemic injury of brain, at least in part by stabilizing HIF-1α, thereby increasing EPO synthesis and activating the cytoprotective Akt signaling cascade.

Recently Ryou *et al*. tested pyruvate's ability to limit rtPA toxicity in a cultured neuronal cell line and primary microvascular endothelial cells.[163] Six and 10 h of oxygen-glucose deprivation produced marked neuronal cell death which was exacerbated by rtPA. Pyruvate (8 mM) prevented cell death in the absence of rtPA, dampened cell death in the rtPA-exposed cells, suppressed rtPA-induced RONS formation, and sharply lowered basal and rtPA-induced MMP-2 content, while inducing Akt and Erk phosphorylation. Interestingly, pyruvate alone or combined with rtPA increased cellular content of monocarboxylate transporter-2 *vs*. the respective pyruvate-free conditions. These results suggested that pyruvate might extend rtPA's therapeutic window by dampening rtPA-induced cytotoxicity; it is essential to test this interaction in intact animals.

Conclusion and perspectives

Cardiac arrest and stroke, two of the leading causes of death and long-term disability in the United States and Europe, heretofore have proven refractory to pharmacological interventions. Extensive preclinical research has identified EPO as a potentially powerful treatment to limit the ischemic damage to the CNS inflicted by these scourges. Unlike agents that failed to protect the CNS in clinical trials, EPO is not a "one trick pony;" it activates several intracellular mechanisms that intervene at multiple steps in the cascade of ischemia-reperfusion injury (Figure 2). However, despite favorable outcomes in early clinical trials, two factors threaten to limit EPO's clinical utility for stroke and cardiac arrest: its potentially dangerous interaction with rtPA inducing hemorrhagic transformation within the cerebral circulation, and the high dosages of EPO required to surmount the BBB.

The brain's intrinsic ability to express and synthesize EPO may afford an alternative strategy: the administration of compounds that promote EPO gene expression within the brain by stabilizing the transcription factor HIF-1, the principal activator of EPO gene expression. Pyruvate offers several advantages as an enhancer of HIF-1-driven EPO expression in the CNS: a natural intermediary metabolite, pyruvate is nontoxic at cerebroprotective dosages; aside from its EPO induction, pyruvate is a physiological antioxidant and energy-yielding, oxidizable fuel; pyruvate is efficiently transferred from the circulation to the brain parenchyma by monocarboxylate transporters within the cerebrovascular endothelium and in the plasma membranes of neurons and glia, delivering it to the sites of ischemia-reperfusion injury and of EPO synthesis; pyruvate is highly water soluble, so that aqueous solutions of concentrated sodium pyruvate suitable for intravenous infusion[164] are readily prepared. Thus, pyruvate therapy may offer a facile means of evoking EPO expression and cytoprotection within the CNS. It should be noted that pyruvate has been shown to be safe and efficacious as an intracoronary intervention in patients with congestive heart failure[165,166] and cardiogenic shock,[167] and as a component of cardioplegia in patients undergoing coronary revascularization on cardiopulmonary bypass.[168]

Potential limitations of pyruvate therapy must be acknowledged. Given HIF-1's fundamental role in promoting survival and growth of solid tumors,[159] protracted pyruvate treatment might impose unacceptable risks in cancer patients. However, this concern would not apply to a single pyruvate treatment for acute CNS ischemia. It has been argued[169,170] that pyruvate may be unsuitable for protracted storage due to its chemical instability. However, pyruvate can be kept indefinitely in powder form and, as noted above, dissolved to high concentrations immediately before its administration. Esterified derivatives of pyruvate, most notably ethyl pyruvate, have been found to be highly stable in aqueous solution, although these compounds are somewhat less soluble than authentic pyruvate,[139] and to suppress systemic inflammation in rat models of endotoxemia[171] and hemorrhagic shock.[172] However, it has been reported that ethyl-pyruvate resuscitation affords no short-term energetic and hemodynamic advantages over standard lactated Ringer's.[173] Moreover, the ability of these pyruvate derivatives to traverse the BBB has not yet been established.

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Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid
BBB	blood brain barrier
cIAP2	c-inhibitor of apoptosis-2
CNS	central nervous system
CPR	cardiopulmonary resuscitation

EPO	erythropoietin
ΔG_{ATP}	Gibbs free energy of ATP hydrolysis
HIF	hypoxia-inducible factor
Keap1	Kelch-like ECH-associated protein 1
MCA	middle cerebral artery
MMP	matrix metalloproteinase
NF-κB	nuclear factor κB
NMDA	*N*-methyl-*D*-aspartate
NOS	nitric oxide synthase (eNOS, endothelial NOS
iNOS	inducible NOS
nNOS	neuronal NOS)
Nrf2	nuclear factor erythroid 2-related factor 2
RONS	reactive oxygen and nitrogen species
ROSC	recovery of spontaneous circulation
rtPA	recombinant tissue plasminogen activator
TIMP	tissue inhibitor of metalloproteinase
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
XIAP	X-linked inhibitor of apoptosis

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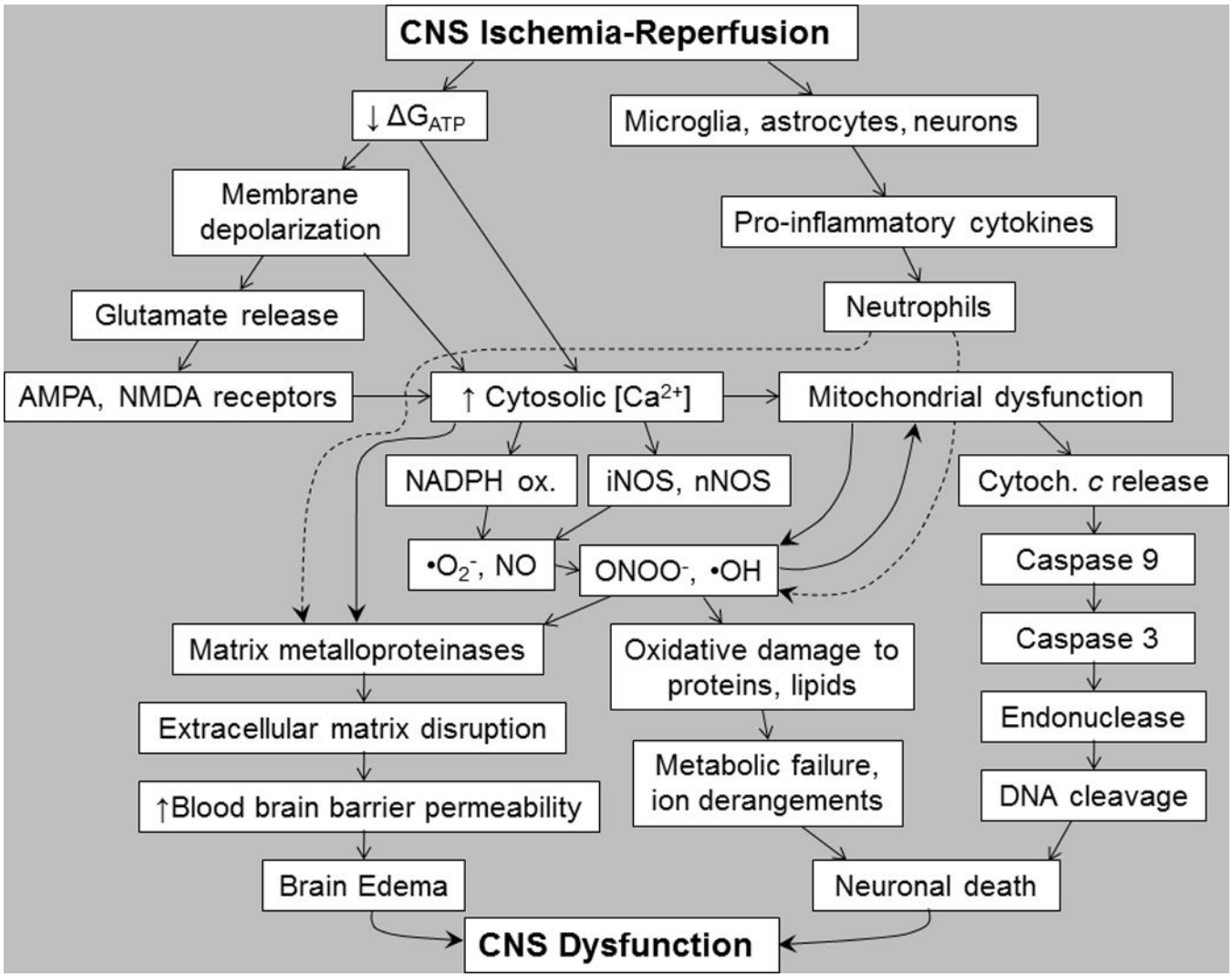

Figure 1. Cascade of injury in ischemic and post-ischemic brain

By interrupting cerebrovascular delivery of energy substrates and O_2, CNS ischemia depletes Gibbs free energy of ATP hydrolysis (ΔG_{ATP}), thus impairing neuronal Ca^{2+} management and provoking excitotoxic glutamate signaling. Subsequent reperfusion triggers intense formation of reactive oxygen and nitrogen species. These compounds and Ca^{2+} overload combine to trigger mitochondrial permeability transition, cytochrome *c* release and energetic collapse, and activate matrix metalloproteinases that degrade the extracellular matrix, allowing neutrophil infiltration in response to pro-inflammatory cytokines and provoking brain edema. See text for details.

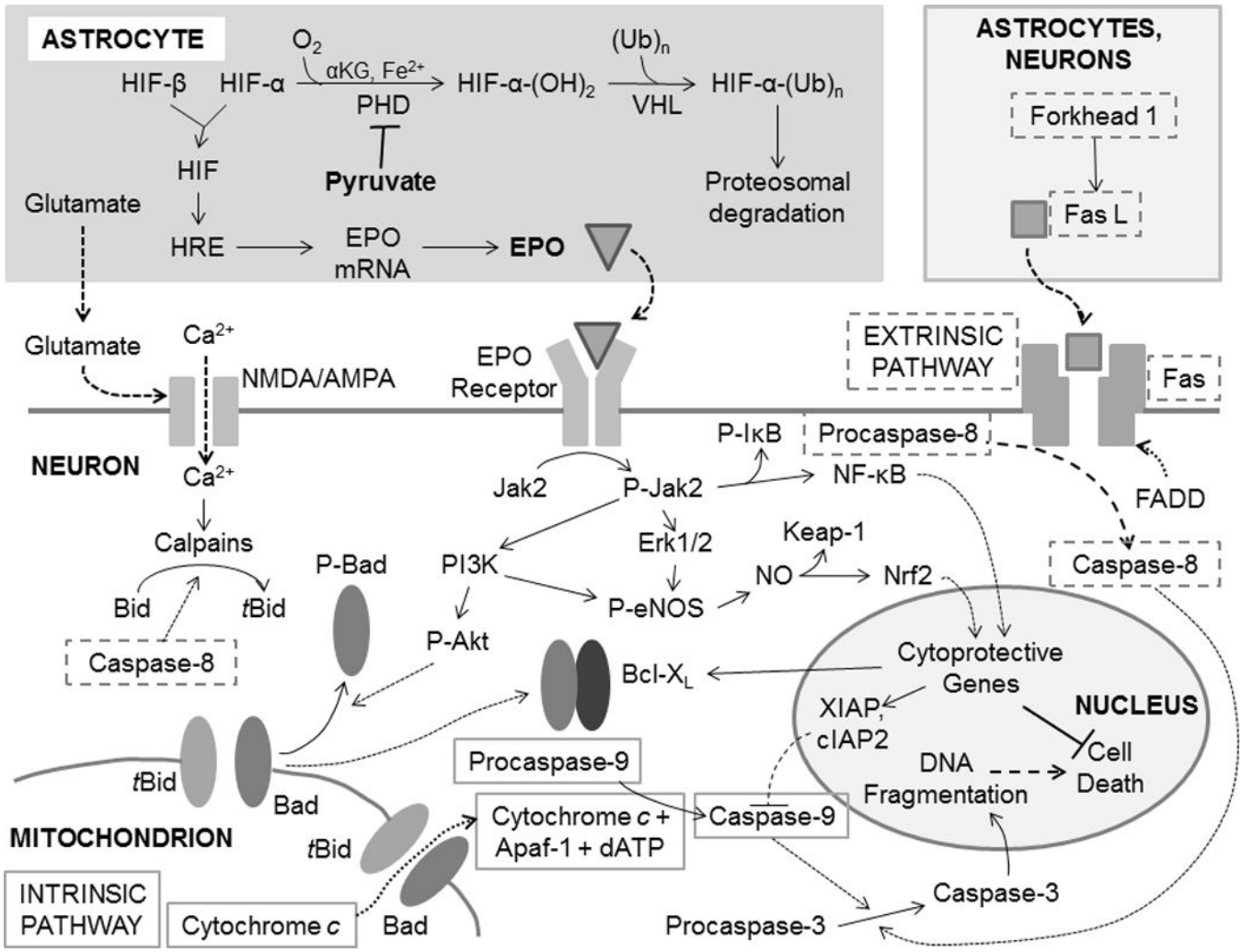

Figure 2. Anti-apoptotic mechanisms of erythropoietin

Ischemia-reperfusion activates intrinsic and extrinsic apoptotic cascades, the elements of which are indicated by solid and broken gray outlines, respectively, which converge on caspase-3 as the common effector. Erythropoietin (EPO) activates anti-apoptotic signaling in neurons by binding its membrane receptors. This event initiates a complex cascade of intracellular signaling events, mediated by protein kinases, that (1) prevent formation of Bad-*t*Bid channels that release cytochrome *c* from mitochondria; (2) blunt the activation of pro-apoptotic caspases; and (3) evoke Nrf2- and NF-κB driven expression of cytoprotective genes that increase neuronal resistance to ischemia-reperfusion stress. Collectively, these mechanisms suppress the intrinsic and extrinsic apoptotic pathways. EPO expression, primarily in astrocytes, is driven by hypoxia-inducible factors (HIF) interacting on hypoxia response elements (HRE) in the promoter regions of EPO and other genes. HIF, in turn, is activated by stabilization of its O_2-regulated α subunit. Pyruvate interferes with HIF-α hydroxylation by prolyl hydroxylase (PHD), thereby preventing proteosomal degradation of the subunit and promoting EPO expression.

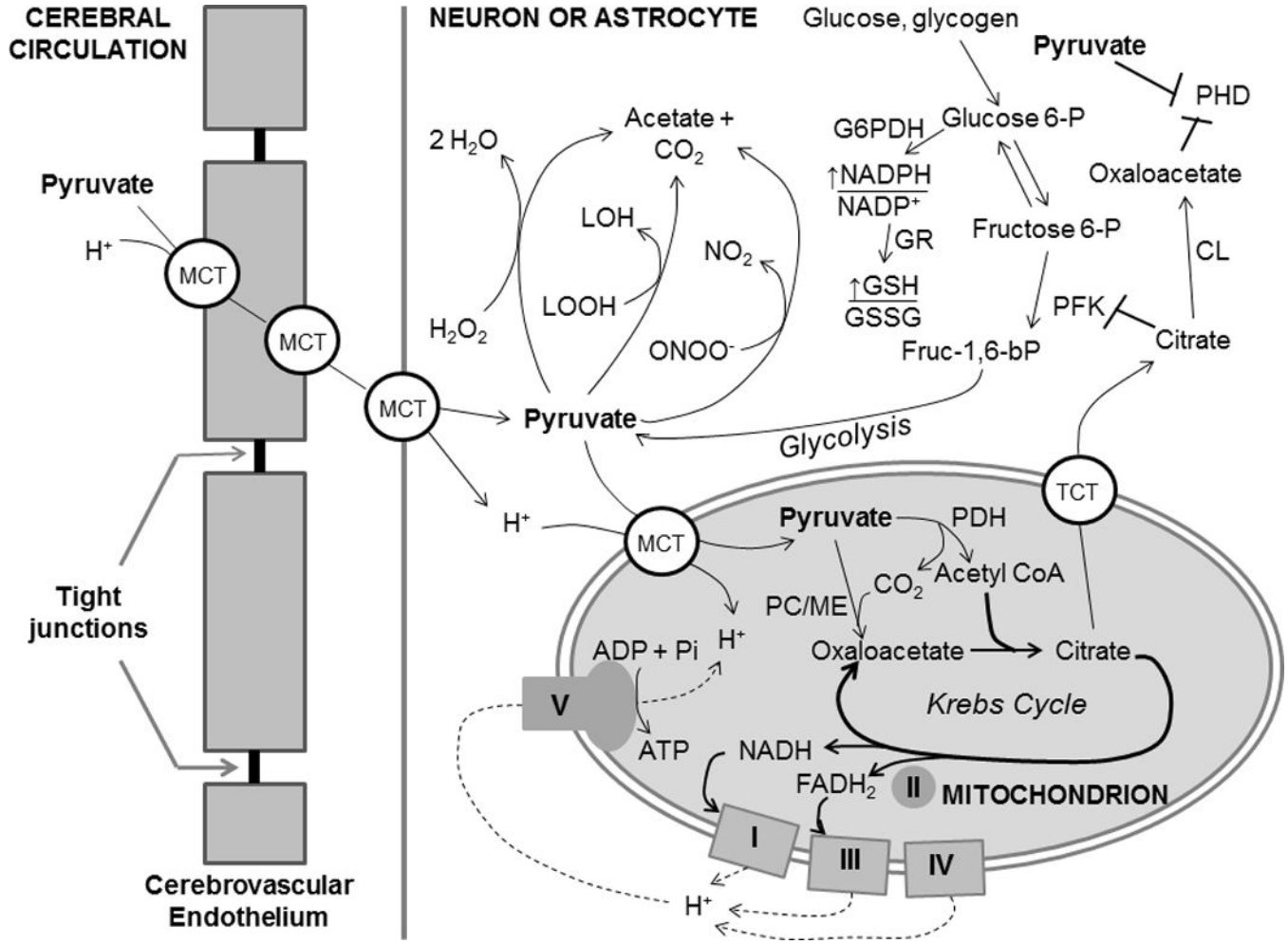

Figure 3. Metabolism and cytoprotective mechanisms of pyruvate in brain

Pyruvate is carried across the cerebrovascular endothelium and cell and mitochondrial membranes within the brain parenchyma my monocarboxylate transporters (MCT). In addition to its induction of EPO expression (Figure 2), pyruvate affords cytoprotection by (1) supporting oxidative metabolism and mitochondrial ATP production; (2) directly detoxifying hydrogen peroxide, lipid peroxides (LOOH) and peroxynitrite; (3) increasing mitochondrial citrate formation, which, when exported to the cytosol by the tricarboxylate transporter (TCT), suppresses phosphofructokinase (PFK) activity, thereby diverting glycolytic flux into the hexose monophosphate shunt, the source of NADPH reducing power by glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase; (4) cytosolic citrate lyase degrades citrate to acetate and oxaloacetate, which, like pyruvate, competitively inhibits prolyl hydroxylase.