Central Nervous System Frontiers for the Use of Erythropoietin

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Recombinant human erythropoietin (r-HuEPO; epoetin alfa) is well established as safe and effective for the treatment of anemia. In addition to the erythropoietic effects of endogenous erythropoietin (EPO), recent evidence suggests that it may elicit a neuroprotective effect in the central nervous system (CNS). Preclinical studies have demonstrated the presence of EPO receptors in the brain that are up-regulated under hypoxic or ischemic conditions. Intracerebral and systemic administration of epoetin alfa have been demonstrated to elicit marked neuroprotective effects in multiple preclinical models of CNS disorders. Epoetin alfa has also been shown to prevent the loss of autoregulation of cerebral blood flow in a model of subarachnoid hemorrhage. The mechanisms of EPO-induced neuroprotection include prevention of glutamate-induced toxicity, inhibition of apoptosis, anti-inflammatory effects, antioxidant effects, and stimulation of angiogenesis. Collectively, these findings suggest that epoetin alfa may have potential therapeutic utility in patients with ischemic CNS injury.

ERYTHROPOIETIN: EFFECTS BEYOND ERYTHROPOIESIS

Erythropoietin (EPO) is a naturally occurring glycoprotein hormone with a primary biologic function of regulating erythropoiesis in response to decreased oxygen delivery to tissues [1–4]. The EPO gene transcription is up-regulated by hypoxia-inducible factor 1 [3, 5]. The recognition of EPO’s key role in the regulation of erythrocyte production led to development of the recombinant human form (r-HuEPO, epoetin alfa) for therapeutic use in the 1980s. Epoetin alfa is identical to the endogenous hormone with respect to structure and biologic activity [6, 7]. Epoetin alfa has been used safely and effectively for over a decade for the treatment of anemia related to a variety of disorders, including chronic renal failure [8], antiretroviral therapy for HIV infection [9, 10], and cancer chemotherapy [11–14], as well as for reduction of allogeneic transfusion before major elective surgery [15–17].

EPO promotes the proliferation and differentiation of erythroid progenitor cells [4]. EPO also promotes the survival of erythroid progenitor cells by preventing programmed (apoptotic) cell death of immature erythroblasts (e.g., colony-forming unit erythroblasts, pro-erythroblasts) [4, 18]. The effects of EPO are mediated via interactions with specific cell surface receptors belonging to the cytokine receptor type 1 superfamily [4]. The molecular pathway of the antiapoptotic effect of EPO appears to be at least partially related to induction of the Bcl-xL regulatory protein (a member of the Bcl-2 family of antiapoptosis regulatory proteins) via activation of the transcription factors GATA-1 [18] and Stat5 [19]. In addition to its erythropoietic effects, EPO recently has been shown to have a number of other properties that may extend the therapeutic applications of r-HuEPO. In particular, there is an increasing body of evidence that EPO has a role in CNS physiology, with neuroprotective effects under conditions of hypoxia or ischemia.

CNS DISTRIBUTION OF EPO RECEPTORS AND PRODUCTION OF EPO

Preclinical studies in rodents and primates have confirmed the presence of EPO and EPO receptors in the brain [20–25]. In the human brain, medium to large neurons in many regions, including the frontal cortex
and hippocampus, exhibit abundant expression of EPO receptors as determined by anti-EPO receptor immunohistochemical detection methods; to date, receptors have been identified primarily in the somata and proximal dendrites, and in capillaries within white matter [25]. EPO receptor immunoreactivity predominates within the astrocytic end feet surrounding the capillaries [25] and on or within the surface of capillary endothelial cells [24–26]. Astrocytes and neurons also have been shown to up-regulate the expression of EPO and/or EPO receptors under experimental hypoxic/ischemic conditions in animal and human brain tissues [20–22, 24, 27–30].

EPO has been detected in the CSF and/or brain of humans who have sustained CNS injury (e.g., traumatic brain injury, asphyxia, intraventricular hemorrhage) [31] or ischemic brain infarcts [30]. In 6 adults with traumatic brain injury, EPO was evident in all CSF samples tested, and CSF levels correlated with the degree of blood-brain barrier dysfunction; however, EPO also was present in CSF when the blood-brain barrier function was normal [31]. In neonates, those with asphyxia or intraventricular hemorrhage had significantly higher CSF concentrations of EPO than controls, whereas those with meningitis and other neurologic diseases did not [32]. However, neonates with intraventricular hemorrhage did not have elevated plasma EPO concentrations, and there was no correlation between CSF and plasma EPO concentrations among those infants treated with epoetin alfa, suggesting that the source of EPO was likely within the CNS [32].

Brain autopsies of patients who had experienced ischemic infarcts or general hypoxic damage revealed that the expression of EPO and EPO receptors was increased compared with brains of neuropathologically normal subjects [30]. In fresh infarcts, the expression of EPO was most evident in vascular elements of the penumbral tissue (area at risk surrounding the infarct core) [30]. EPO was evident in vascular endothelium even within the infarct core [30]. Expression of EPO receptors in the peri-infarct regions was most evident in neuronal somas and processes but also was strong in broken neuronal filaments in the infarct core [30]. This increased receptor expression may indicate a local response to ischemic injury whereby EPO is directed toward neurons at risk of death [30]. In acute hypoxic brain damage, there was strong expression of EPO in vascular endothelium with expression of EPO receptors in neuronal somas [30]. In older infarcts or older hypoxic damage, EPO and the EPO receptor were strongly expressed in reactive astrocytes and, to a lesser extent, in blood vessels surrounding infarcted tissue [30]. At later times after infarct, EPO and EPO receptor expression apparently shift to reactive astrocytes, cells that may be involved in tissue repair. In addition, in the event of repeated episodes of hypoxia/ischemia, the ability of astrocytes to up-regulate EPO production may provide a rapidly mobilizable pool of EPO [30].

Collectively, in vitro and in vivo findings suggest that local or systemic hypoxia is a stimulus for increased EPO production and EPO receptor expression in the CNS and that elevated EPO concentrations are not merely a nonspecific marker for CNS injury [32]. However, there may be other potential stimuli for brain EPO production, including hypoglycemia [5, 29] and inflammation [33]. Stimulation of brain EPO synthesis might occur under any condition characterized by a relative ATP deficiency associated with an increase in metabolic stress [33, 34].

**EPO CNS TRANSPORT**

Although it had been widely held that large proteins such as EPO cannot cross the blood-brain barrier because of their high extent of glycosylation and molecular weight, a number of large proteins have been shown to be transported into the CNS through receptors on the surface of brain capillary endothelial cells [35–39]. Recent evidence suggests that EPO can be transported into the CNS. Five hours after intraperitoneal (ip) injection into mice, biotinylated epoetin alfa (5000 U/kg body weight) reaction product was detected surrounding the capillary endothelium by the streptavidin-peroxidase system [25]. Visualizations of brain sections showed that the peroxidase reaction product had surrounded capillary lumens and had extended into brain parenchyma a distance 3–4 times that of the capillary wall thickness [25]. After 17 h, the labeled product was no longer observed around the capillaries but was localized to scattered neurons [25]. A progressive increase in concentrations of EPO in the CSF was observed after a 1-h lag time, with peak CSF levels occurring 3.5 h after administration (figure 1) [40]. The transport mechanism into the CNS appeared to

![Figure 1. CSF concentrations of erythropoietin (EPO) after injection of 5000 U/kg ip in rats (n = 7). *P < .01; †P < .003. Reproduced with permission from Cerami A. Semin Hematol 2001;38(Suppl 7):33–9 [40].](http://cid.oxfordjournals.org/Downloadedfrom)
Neuroprotective effects of EPO and epoetin alfa have been demonstrated recently in a variety of rodent models of hypoxic/ischemic CNS disorders (Table 1).

**Global and focal ischemia models.** The neuroprotective effect of EPO and epoetin alfa has been demonstrated in vitro [23, 29, 43] and in vivo in rodent stroke models [24, 25, 41, 42, 44]. In cultured rat cortical neurons, exposure to epoetin alfa or recombinant mouse EPO significantly reduces hypoxia-induced [29], hypoxia plus glucose deprivation–induced [43], and glutamate-induced [23, 42] cell death.

Several studies in rodent stroke models demonstrated neuroprotective effects of epoetin alfa when administered by direct cerebroventricular infusion [24, 41, 42]. Sakana et al. [42] demonstrated that cerebroventricular infusion of epoetin alfa in gerbils prevented ischemia-induced learning disability and hippocampal CA1 neuron damage in a dose-dependent fashion. Electron microscopy confirmed that synapses in the hippocampal CA1 region were significantly more numerous among epoetin alfa–treated gerbils than among vehicle-treated gerbils [42]. The hypothesis that EPO is important for neuronal survival in the CNS was tested with an ischemia-induced model of neuronal damage in these animals. A soluble form of the EPO receptor (sEPOR) comprising the extracellular ligand-binding region of the receptor was infused into the cerebral ventricles after a 2.5-min ischemic insult. Alternatively, an inactive form of the EPO receptor (dsEPOR) was infused into another group of gerbils after the same ischemic insult. The animals that had received sEPOR had neuronal damage, evidenced by a significant reduction in response latency time and a significant decrease in hippocampal CA1 neuron density [42]. Animals that received inactivated dsEPOR failed to exhibit neuronal damage [42].

In a related study in stroke-prone, spontaneously hypertensive rats with a permanent occlusion of the middle cerebral artery (MCA), epoetin alfa or vehicle control was infused directly into the cerebral ventricles [41]. Epoetin alfa treatment for 28 days significantly alleviated place navigational disabilities induced by ischemia and prevented secondary degeneration of thalamic neurons compared with vehicle controls in MCA-occluded rats [41]. In another study, recombinant mouse EPO or vehicle control was administered to mice via intracerebroventricular injection 24 h before focal ischemia was induced by permanent occlusion of the left MCA [24]. Pretreatment with recombinant mouse EPO 24 h before occlusion led to a 47% reduction in infarct volume compared with control [24].

More recent studies have demonstrated that systemic administration of epoetin alfa also can decrease focal ischemia–induced neuronal damage [25, 44]. By use of a rat stroke model, right frontal cortical ischemia was induced by permanent occlusion of the right middle cerebral and carotid arteries, followed by a reversible 1-h occlusion of the left carotid artery [25]. Epoetin alfa (up to 5000 U/kg) or vehicle control was injected ip 24 h before; simultaneously with; or 3, 6, or 9 h after MCA occlusion [25]. Administration of epoetin alfa 24 h before and up to 6 h after occlusion produced significant reductions in infarct volume compared with vehicle-treated rats [25]. The neuroprotective effect of epoetin alfa began to diminish by 6 h after occlusion and was no longer evident when the drug was provided 9 h after occlusion [25]. The minimum effective dose of epoetin alfa administered at the time of occlusion was ~450 U/kg [25]. These data suggest that there is a therapeutic window of 6 h after an ischemic insult during which systemically administered r-HuEPO may provide neuroprotection [25].

In another study that used the same rat model of focal ischemia to investigate the mechanism involved, similar results were obtained [44]. Administration of epoetin alfa 5000 U/kg ip at the time of occlusion produced an ~75% decrease in infarct volume after 24 h compared with vehicle-treated rats [44]. Histologic examination revealed that although all rats had a small ischemic core, only vehicle-treated rats had widespread tissue infarction adjacent to the ischemic core [44]. In this ischemic penumbra, epoetin alfa–treated rats exhibited a marked absence of inflammatory cells [44]. The short-term effects appeared to be related to inhibition of neuronal apoptosis, but epoetin alfa also was shown to be trophic in cultured neuronal cells [44].

**Neurotrauma model.** Head trauma produces a dynamic process characterized by a cascade of metabolic, cellular, and molecular events [49]. These include diffuse axonal injury, ischemia, metabolic abnormalities, excitotoxicity, and oxidative...
stress [49]. These processes continue for unpredictable periods after the initial injury [49]. On the basis of the neuroprotective effect of epoetin alfa in the rat stroke model, the drug was evaluated in a mouse model of blunt trauma [25]. Mice received ip epoetin alfa (5000 U/kg) or vehicle control (saline) 24 h before or 0, 3, or 6 h after the delivery of a calibrated blow to the intact calvaria [25]. A 3-mm-diameter stainless steel piston delivered a blow 2 mm caudal and 2 mm ventral to the bregma (the craniometric point at the junction of the coronal and sagittal sutures of the skull) [25]. Epoetin alfa administration was continued once daily for an additional 4 days [25]. Examination of brain sections 10 days after the delivery of a 4-m/s blow with a 2-mm displacement revealed that epoetin alfa–treated animals had a significantly smaller volume of injury than those who received saline [25]. There was a similar degree of protection across the different epoetin alfa administration times [25]. Histologic examination revealed a dense area of mononuclear inflammatory cells immediately surrounding the necrotic core among mice receiving saline [25]. In contrast, epoetin alfa–treated mice had markedly reduced inflammatory infiltrate [25].

**Experimental autoimmune encephalomyelitis model.** The unexpected anti-inflammatory effect observed in the blunt trauma model provided a rationale for testing whether epoetin alfa could reduce nervous system inflammation in a rat model of experimental autoimmune encephalomyelitis (EAE) [25]. EAE was induced by immunization of female Lewis rats with guinea pig myelin basic protein in complete Freund’s adjuvant [25]. In this model, symptoms develop by day 10 among immunized rats, with a peak degree of paralysis evident by day 12 [25]. Administration of epoetin alfa 5000 U/kg ip once daily was initiated 3 days after immunization and continued until day 18 [25]. Epoetin alfa–treated rats demonstrated a significantly delayed onset and decreased severity of symptoms compared with control rats [25]. Continued observation for 3 weeks after discontinuation of epoetin alfa revealed no rebound of symptoms, as is typically seen after discontinuation of other anti-EAE agents such as glucocorticoids or IFN-β [50].

**Kainate-induced seizure model.** Kainate is an analog of glutamate, and the kainate-induced seizure model is a common method for assessing excitotoxicity. Epoetin alfa 5000 U/kg or saline control was administered ip to mice 24 h before ip administration of kainate 20 mg/kg [25]. Mice treated with epoetin alfa experienced a significant delay in the onset of status epilepticus and a markedly reduced motor involvement [25]. In addition, there was a 45% reduction in mortality and a significant increase in survival time among epoetin alfa–treated mice [25]. Administration of epoetin alfa was effective in increasing mean survival time when administered as early as 3 days before the induction of seizures [25].

**Subarachnoid hemorrhage model.** The most frequent cause of subarachnoid hemorrhage (SAH), excluding head trauma, is rupture of a saccular aneurysm [51]. Subsequent cerebrovascular dysfunction is an important contributor to the delayed development of cerebral ischemia, the major cause of delayed morbidity and mortality in patients who survive the initial bleeding of an SAH episode [51]. Specifically, the development of vasoconstriction is associated with disruption of endothelium-dependent, nitric oxide (NO)–mediated vasodilation, possibly as a result of reduced amounts of endothelial-derived NO in cerebral vessels.

It was postulated that epoetin alfa may have a beneficial effect on the initial inflammatory cerebrovascular dysfunction that leads to ischemic neuronal damage [47]. This hypothesis was evaluated in a model of SAH in which 0.07 mL of autologous blood or sham saline was injected into the cisterna magna of male Sprague-Dawley rats [47]. Epoetin alfa (400 U/kg sc) or vehicle control was administered immediately after the induction of SAH or sham operations in 4 groups of rats: (1) sham operation plus vehicle; (2) sham operation plus epoetin alfa; (3) SAH plus vehicle; and (4) SAH plus epoetin alfa [47]. Forty-eight hours after blood or saline injection, cerebral blood flow autoregulation was evaluated by the intracarotid 133Xe method [47]. After injection of 133Xe, activity was measured with a sodium-iodide (NaI) crystal, and cerebral blood flow was calculated from the initial slope of the washout curve after correction for background [47]. In a normal relationship between mean arterial blood pressure (MABP) and cerebral blood flow, a threshold MABP is achieved above which further increases in MABP do not result in changes in cerebral blood flow, and the autoregulation curve reaches a plateau. This profile was observed in rats receiving sham operation plus vehicle (figure 2, Group A) [47]. Administration of epoetin alfa did not affect the autoregulatory threshold in the sham operation plus epoetin alfa control rats (figure 2, Group B) [47]. As expected, in vehicle-treated rats with SAH a plateau of cerebral blood flow was not established and there was a linear relationship between cerebral blood flow and MABP, indicating that autoregulation of cerebral blood flow was abolished (figure 2, Group C) [47]. However, in the SAH plus epoetin alfa group, epoetin alfa prevented the loss of autoregulation of cerebral blood flow in rats with SAH (indistinguishable from the other 2 control groups with intact autoregulation) (figure 2, Group D) [47]. These data suggest that early activation of endothelial EPO receptors may be a potential therapeutic strategy to protect against cerebrovascular perturbations after SAH [47]. Further studies are needed to determine how the beneficial effects of epoetin alfa on cerebral blood flow autoregulation relate to brain ischemic damage and outcome after SAH [47]. However, the results confirm the work of others, also demonstrating a beneficial effect of epoetin alfa in experimental models of SAH [45, 46].

**Ischemic spinal cord injury model.** To investigate whether
Figure 2. Effect of epoetin alfa on cerebral blood flow (CBF) autoregulation. Four groups of Sprague-Dawley rats received an injection of either blood (subarachnoid hemorrhage [SAH] induction) or of sham control saline into the cisterna magna. These groups then received a single subcutaneous dose of either epoetin alfa or vehicle control. Reproduced with permission from Springborg JB, Ma XD, Rochat P, et al. A single subcutaneous bolus of erythropoietin normalizes cerebral blood flow autoregulation after subarachnoid haemorrhage in rats. Br J Pharmacol 2002; 135:823–9 [47]. MABP, mean arterial blood pressure.

Epoetin alfa might exert a neuroprotective effect during spinal cord ischemic injury in vivo, ischemia was induced in rabbits by occlusion of the abdominal aorta for 20 min [48]. Immediately after release of the occlusion, animals received either normal saline or 350, 800, or 1000 U/kg of epoetin alfa [48]. In the saline-treated animals, extensive tissue necrosis and injury was present throughout the spinal cord. However, in epoetin alfa–treated animals, although moderate neurologic disability was observed, histologic evidence of injury was often lacking [48]. In addition, staining with terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL), an indicator of motor neuron injury, was evident in sections of spinal cords from saline-treated animals. In contrast, ischemic spinal cords of animals that had received epoetin alfa were generally devoid of TUNEL reaction product [48]. One hour after reperfusion, all epoetin alfa–treated animals demonstrated better neurologic scores than the saline control group, with the group receiving 800 U/kg having significantly better residual motor function than the saline control group. At 24 and 48 h after reperfusion, all epoetin alfa–treated groups exhibited similar and significantly improved neurologic function compared with the saline control group, whose neurologic score remained unchanged. The results suggest that clinical studies to evaluate the efficacy of epoetin alfa for the treatment of spinal cord ischemia in patients undergoing thoracoabdominal aortic surgery are warranted [48].

**POTENTIAL MECHANISMS OF NEUROPROTECTION**

The mechanisms of the neuroprotective effects of EPO appear to be complex and possibly numerous (table 2). One potential mechanism involves glutamate, a primary excitatory neurotransmitter in the mammalian CNS and a key mediator of neuronal injury [23]. Excessive glutamate concentrations, as a result of hypoxia or hypoglycemia, are believed to be primarily responsible for neuronal death [23]. Epoetin alfa and recombinant mouse EPO have been shown to protect against glutamate- or N-methyl-D-aspartate- (NMDA, a type of glutamate receptor) receptor-induced toxicity [23, 24, 42].

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<th>Table 2. Potential neuroprotective mechanisms of erythropoietin.</th>
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<td>Prevention of glutamate toxicity [23, 24, 42]</td>
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<td>Reduction of apoptosis [44, 52]</td>
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<td>Anti-inflammatory effects [25, 42]</td>
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<td>Antioxidant effects [42, 53–55]</td>
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<td>Stimulation of angiogenesis [56]</td>
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example, exposure of mouse neocortical neurons in vitro to recombinant mouse EPO for 24 h before exposure to NMDA produced a 65%–79% reduction in NMDA-induced neuronal damage compared with control [24]. The molecular mechanism of this effect may be related to EPO-induced modulation of glutamate-induced intracellular Ca++ flux [23], but this has not been clearly established [42]. EPO also protects against neuronal death induced by NO-generating agents [42]. Because glutamate toxicity is mediated in part by NO, EPO’s neuroprotective effect may include the reduction of NO-mediated free radical formation or antagonism of their toxicity [42]. EPO may interact with inducible NO synthase (iNOS)-induced formation of NO at the transcriptional level [57–59].

Epoetin alfa also has been demonstrated to be a potent inhibitor of neuronal apoptosis induced by various stimuli such as hypoxia, nutrient growth factor deprivation, and kainate exposure [44]. The antiapoptotic effect required pretreatment with epoetin alfa, suggesting that the effect is mediated via induction of gene expression [44]. Epoetin alfa appears to modulate distinct signaling cascades in neurons that have been previously characterized in hematopoietic cell lines, and these pathways appear critical to the neuroprotective effects [44].

Another potential neuroprotective mechanism of EPO is its anti-inflammatory properties. Epoetin alfa markedly decreases inflammatory infiltrate in a mouse model of blunt trauma [25]. Furthermore, the efficacy of epoetin alfa in reducing the severity of manifestations in the rat EAE model is consistent with an anti-inflammatory action, although the immune response also appears to be affected [25]. Recent studies demonstrated that epoetin beta reduced the response of vascular smooth muscle cells to inflammatory cytokines in vitro [57] and that epoetin alfa increased survival, enhanced MABP, and improved vascular dysfunction in a rat model of circulatory shock [59]. Both in vitro and in vivo, EPO inhibits cytokine-stimulated activity of iNOS as manifested by a reduction in nitrite production and decreased expression of iNOS mRNA and protein [57–59].

Because EPO protects against neuronal death induced by NO-generating agents [42] and inhibits iNOS activity stimulated by cytokines [57–59], it has been postulated that EPO may up-regulate the expression of antioxidant factors such as superoxide dismutase, glutathione peroxidase, and catalase in neurons and erythrocytes [42, 55]. Other evidence for EPO’s antioxidant properties stems from observations that in vitro epoetin alfa protects against erythrocyte membrane damage caused by hydroxyl radicals [53] and from reduced lipid peroxidation and enhanced antioxidant activities in patients with anemia of chronic kidney disease treated with epoetin alfa [54, 55]. EPO could directly serve as an antioxidant by scavenging oxyradicals and indirectly by stimulating other antioxidant-defense mechanisms [44, 53]. Further studies of EPO-induced up-regulation of neuronal gene expression are needed to identify target molecules involved in EPO’s antioxidant actions [42].

Epoetin alfa also has demonstrated proangiogenic properties in cultured human vascular endothelial cells and the ability to stimulate neovascularization in the chick embryo chorioallantoic membrane assay [56]. The ability to directly interact with endothelial cells and elicit an angiogenic response may be a mechanism to increase microcirculation and tissue oxygenation in the ischemic area in the initial period after a stroke, leading to a decrease in hypoxia and minimal damage to the core region.

**SUMMARY**

It is now evident that EPO has a wider physiologic role than was once believed, particularly in the CNS. The role of this hormone may go beyond its ability to stimulate erythropoiesis by inhibiting apoptosis. EPO may exert its beneficial effects both systemically or locally, as an agent with a broader physiologic role under conditions of hypoxia or ischemia. EPO and EPO receptors have been identified throughout the human brain. Importantly, the expression of EPO and EPO receptors is up-regulated in humans who have sustained CNS injury or an ischemic insult. Activation of endothelial EPO receptor expression and local induction of EPO production may be adaptive mechanisms designed to prevent further damage to neurons and to provide beneficial effects for neurons at risk of dying [30]. Epoetin alfa has demonstrated neuroprotective effects in multiple preclinical models of CNS disorders, including ischemia/hypoxia, neurotrauma, EAE, and kainate-induced seizures. Epoetin alfa also recently has been shown to normalize autoregulation of cerebral blood flow in a model of SAH. Importantly, epoetin alfa appears to provide the same degree of neuronal protection whether administered systemically or directly into the brain.

The body of preclinical findings provides a compelling rationale for investigating the use of epoetin alfa in a variety of human CNS disorders. Such trials are being initiated. A randomized, double-blind clinical trial evaluating the effect of epoetin alfa (administered intravenously) in patients with acute stroke is currently underway in Germany (the Göttingen EPO-Stroke Trial). Data from the first phase 2 trial indicate that epoetin alfa has substantial beneficial effects in these patients [60]. As the first clinical trial designed to examine the use of epoetin alfa in patients with CNS ischemia, further reports on the results are awaited with considerable interest [60].

**References**

47. Springborg JB, Ma XD, Rochat P, et al. A single subcutaneous bolus


