

Brain-derived erythropoietin protects from focal cerebral ischemia by dual activation of ERK-1/-2 and Akt pathways

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SPECIFIC AIMS

The hematopoietic growth factor erythropoietin (Epo) exhibits neuroprotective activity under ischemic conditions in brain, retina, and spinal cord. Epo's neuroprotective properties were confirmed in a clinical trial in humans, which demonstrated that Epo indeed reduces infarct volume and improves functional recovery. These findings raised new hopes that neuroprotection is feasible in humans. Unfortunately, little was known about the signaling mechanisms mediating Epo's neuroprotective actions *in vivo* after stroke. The aims of this study were to 1) test Epo's neuroprotective efficacy after focal cerebral ischemia in a transgenic mouse line, termed tg21, which exhibits elevated Epo levels in the brain but not the blood and 2) elucidate the signaling mechanisms responsible for Epo's neuroprotective function.

PRINCIPAL FINDINGS

1. Human Epo is expressed in the cerebral cortex and striatum of tg21 mice

Neuronal expression of Epo in our mouse line is driven by a platelet-derived growth factor (PDGF) B-chain promoter. To determine whether our tg21 mouse expresses human Epo in the brain, we performed radio immunoassays with tissue samples obtained from the cerebral cortex and underlying striatum, the main structures forming the middle cerebral artery (MCA) territory. Compared with wild-type (WT) animals, Epo levels were elevated ~2.1-fold in tg21 mice (50.3 ± 4.5 vs. 23.7 ± 2.8 mU Epo/mg; $P < 0.05$).

2. Brain neurons express the Epo receptor

To elucidate whether brain neurons of WT and tg21 mice express the Epo receptor at comparable levels, immunohistochemical stainings were prepared. Immunohistochemical analysis revealed a robust expression of Epo receptor on NeuN+ neurons both in the

cerebral cortex and striatum. Expression levels of Epo receptor did not differ between WT and tg21 mice.

3. The Epo receptor-associated protein JAK-2 is activated by human Epo

To find out whether human Epo activates JAK-2, we performed Western blot analyses for the phosphorylated (i.e., activated) JAK-2 protein. Increased phosphorylation levels of JAK-2 in the brain of tg21 mice indicated that JAK-2 activation took place in the presence of human Epo, confirming that the Epo receptor is functional in tg21 mice.

4. Neuronal expression of Epo reduces infarct volume and brain swelling, and also improves neurological deficits

To elucidate how human Epo influences ischemic brain injury, WT and tg21 mice were subjected to 90 min intraluminal MCA occlusion. This procedure results in reproducible brain infarcts involving the cortex and striatum, which develop over 24 h after reperfusion onset. Cerebral blood flow was controlled by laser Doppler flowmetry, which did not reveal any differences between WT and tg21 mice. Experiments showed that transgenic Epo reduced infarct volume by ~84% (from 61.5 ± 8.0 mm³ in WT to 9.6 ± 3.3 mm³ in tg21 mice), diminished brain edema (from $28 \pm 3\%$ to $7 \pm 6\%$ of contralateral), and ameliorated neurological deficits (deficit score 1.0 ± 0.5 in WT vs. 2.2 ± 0.7 in tg21 mice). tg21 animals exhibited small striatal infarcts that almost completely spared the cerebral cortex. Additional latex angiographies demonstrated that the reduction in infarct size was not attributed to differences in the cerebrovascular anatomy between WT and tg21 animals.

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5. Epo decreases disseminate neuronal injury and reduces neuronal NO synthase (NOS)-1 and -2 expression

To determine whether Epo also reduces disseminate neuronal injury, WT and tg21 animals were subjected to 30 min MCA occlusion. This procedure results in delayed neuronal death in the striatum, but not cortex. Cerebral blood flow was controlled by laser Doppler flowmetry, which did not reveal any differences between WT and tg21 mice. TUNEL and immunohistochemical stainings showed that transgenic Epo reduced the density of DNA-fragmented (Fig. 1A, B) and activated caspase-3(+) (Fig. 1A, C) cells in the ischemic striatum. Attenuation of brain injury was associated with reduced NOS-1 (Fig. 1A, D) and NOS-2 (Fig. 1A, E) levels in NeuN(+) neurons.

6. Epo activates ERK-1/-2, Akt, JNK-1/-2, and Bcl-X_L signaling

To analyze cell signaling pathways responsible for Epo's neuroprotection, we prepared Western blots with tissue samples from mouse striatum. Blots of nonischemic brain samples showed that total levels of ERK-1/-2, Akt, and JNK-1/-2 did not differ between tg21 and WT animals (Fig. 2). Phosphorylated ERK-1/-2, Akt, and JNK-1/-2 levels were higher and Bcl-X_L expression was slightly lower in tg21 than WT mice (Fig. 2).

Upon ischemia, phosphorylated ERK-1/-2 and JNK-1/-2 further increased in tg21 animals but remained unchanged or decreased in WT mice (Fig. 2). Akt phosphorylation remained high in tg21 animals (Fig. 2). Bcl-X_L expression increased above levels of WT mice (Fig. 2). Our data suggested a role of ERK-1/-2, Akt, and Bcl-X_L in Epo's neuroprotective function.

7. Epo's neuroprotective function in vivo requires dual activation of ERK-1/-2 and Akt

To define whether ERK-1/-2 or Akt signaling pathways are responsible for Epo's neuroprotection, we applied signal transduction inhibitors PD98059 (ERK-1/-2) and Wortmannin (Akt) into intracerebroventricular space. Both signal inhibitors abolished the tissue neuroprotection induced by Epo (Fig. 1A–C). Our data indicate that Epo's neuroprotective activity depends on the dual activation of both pathways (Fig. 3).

8. Inhibition of NO synthases by human Epo does not depend on ERK-1/-2 and Akt

To determine whether ERK-1/-2 or Akt mediates the inhibition of NOS-1 and -2 by human Epo, we prepared immunohistochemical stainings from animals receiving PD98059 and Wortmannin. Neither ERK-1/-2 (PD98059) nor Akt (Wortmannin) inhibition restored NOS-1 and -2 levels in ischemic neurons (Fig. 1A, D, E). Our data indicate that the NOS-1 and -2 inhibition by Epo occurs independent of ERK-1/-2 and Akt.

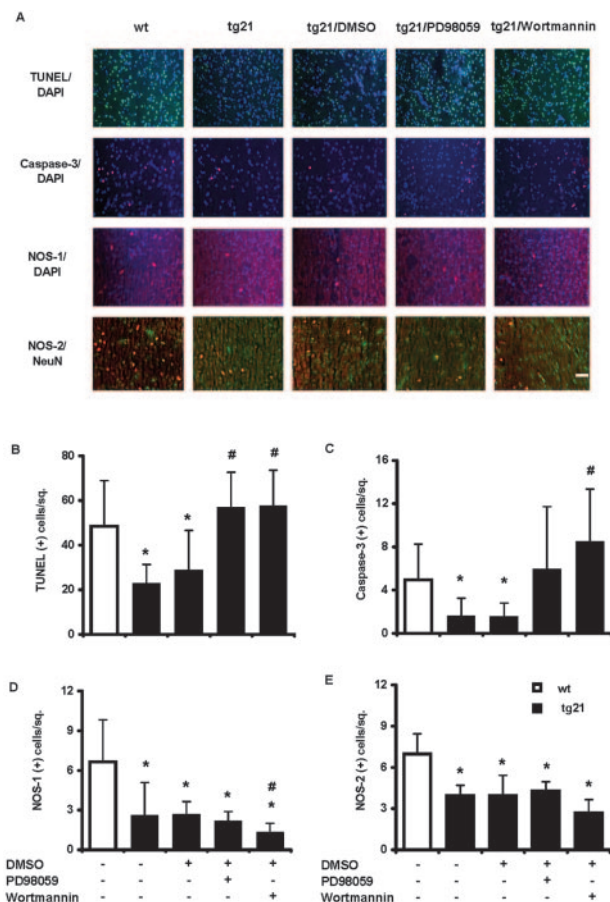
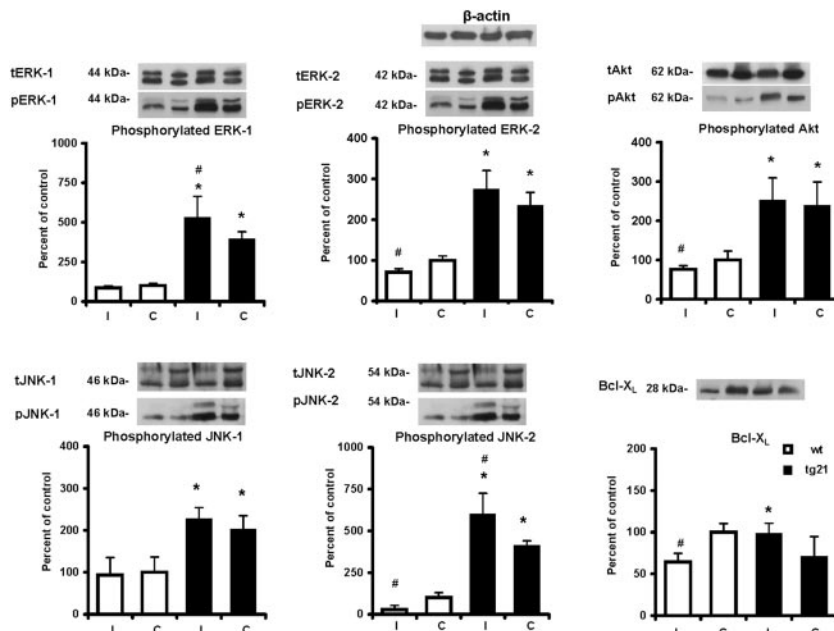


Figure 1. Human Epo protects against focal cerebral ischemia by dual activation of ERK-1/-2 and Akt. TUNEL stainings as well as immunohistochemistries for activated caspase-3, NOS-1, and NOS-2 were evaluated in the ischemic mouse striatum of WT and tg21 mice subjected to 30 min MCA occlusion (A). Densities of DNA fragmented (B), activated caspase-3+ (C), NOS-1+ (D), and NOS-2+ (E) cells were quantified. Animals were either untreated or received intracerebroventricular injections of the solvent DMSO, which was given in the presence or absence of the ERK-1/-2 inhibitor PD98059 and PI3K/ Akt inhibitor Wortmannin. Note that human Epo reduces delayed neuronal death in the striatum (A, B), at the same time decreasing activated caspase-3 levels (A, C) and reducing NOS-1 (A, D) and NOS-2 (A, E) expression in ischemic neurons. Antagonization of either ERK-1/-2 and Akt pathways abolishes tissue protection induced by Epo (A–C). On the other hand, inhibition of ERK-1/-2 and Akt does not reverse the inhibition of NOS-1 and -2 in tg21 mice (A, D, E), indicating that NOS-1 and -2 inhibition by Epo occurs in an ERK-1/-2 and Akt independent manner. Fluorescence colors in A: TUNEL, NeuN: green (FITC); caspase-3, NOS-1/-2: red (Cy-3); DAPI: purple; superimposed: TUNEL/DAPI: bright green, caspase-3/DAPI//NOS-1/DAPI: pink; NOS-2/NeuN: yellow. Data are mean ± SD. values (n=6 animals/ group). *P < 0.05 compared with WT mice; #P < 0.05 compared with DMSO-treated mice. Bar, 200 μm.

CONCLUSIONS AND SIGNIFICANCE

Using the transgenic mouse line tg21, which predominantly expresses human Epo in the CNS, we demonstrate that Epo protects against focal cerebral ischemia

Figure 2. Human Epo elevates ERK-1/-2, Akt and JNK-1/-2 phosphorylation and stimulates Bcl-X_L expression after focal cerebral ischemia induced by 30 min MCA occlusion. Western blots with brain tissue homogenates of WT and tg21 mice obtained from the ischemic striatum are presented. Phosphorylated (but not total) levels of ERK-1/-2, Akt and JNK-1/-2, as well as Bcl-X_L expression are increased by human Epo. Our data indicate that ERK-1/-2, Akt and Bcl-X_L are involved in Epo's neuroprotective function. Data are mean \pm SD. Values ($n=3$ different samples/group) normalized with corresponding blots for β -actin are shown. I, ischemic; C, contralateral nonischemic. * $P < 0.05$ compared with WT mice; # $P < 0.05$ compared with nonischemic tissue.



in vivo via ERK-1/-2 and Akt signaling. Our data were obtained using 1) in vivo experiments in which brain injury was evaluated following intraluminal MCA occlusions of two different durations either leading to brain infarction (90 min) or disseminate neuronal injury (30 min); 2) histochemical studies using brain tissue obtained from these animals where we examined unphosphorylated and phosphorylated (i.e., activated) signal transduction factors, pro- and antiapoptotic proteins, as

well as NOS-1 and -2; and 3) additional in vivo experiments in which signal transduction inhibitors for ERK-1/-2 (PD98059) and Akt (Wortmannin) were injected into the intracerebroventricular space and in which effects of transgenic Epo on ischemic brain injury were evaluated. Our study shows a marked neuroprotective action of Epo that was superior to other growth factors previously assessed in our laboratory.

Earlier data of our group in a model of optic nerve transection indicated that Epo's protective function in retinal ganglion cells, which are also CNS neurons, is driven predominantly by the ERK-1/-2 pathway. Thus, we hypothesized that ERK-1/-2 may also mediate neuroprotection after ischemic stroke. It is noteworthy that Epo's neuroprotective activity after stroke requires dual activation of ERK-1/-2 and Akt. In ischemic neurons, Akt signaling exhibits a strong stabilizing influence on mitochondrial function, which plays a crucial role in ischemic injury. Upon phosphorylation of Bad, activated Akt releases Bcl-X_L inside the mitochondria that subsequently prevents formation of the mitochondrial permeability transition pore. Bcl-X_L also inhibits the secretion of cytochrome c into the cytosol, thereby impeding activation of executioner caspases.

After recent clinical studies indicating that Epo protects against ischemic stroke in humans, chances appear favourable that it should soon be possible to establish a neuroprotection therapy for stroke patients based on Epo. The >80% reduction of infarct volume in ischemic tg21 animals confirms that Epo is indeed a potent neuroprotectant. Based on our findings presented here, the high potency of Epo may be attributed on its combined action on ERK-1/-2, Akt, and possibly also NOS-1/-2.

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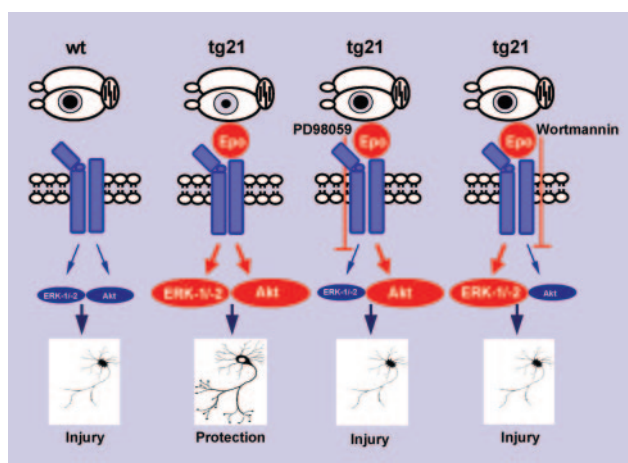


Figure 3. Epo protects against ischemic brain injury by dual activation of ERK-1/-2 and Akt pathways. In the brain of ischemic WT mice, ERK-1/-2 and Akt activity are low. As a consequence, the ischemic neurons die. In tg21 mice constitutively expressing human Epo, on the other hand, ERK-1/-2 and Akt signaling are activated and neurons remain viable. Inhibition of either ERK-1/-2 (with PD98059) or Akt (with Wortmannin) pathways abolishes the protection effects of Epo, indicating that Epo's neuroprotective action depends on the simultaneous activation of both pathways.