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The nonerythropoietic asialoerythropoietin protects against neonatal hypoxia-ischemia as potently as erythropoietin

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Abstract

Recently, erythropoietin (EPO) and the nonerythropoietic derivative asialoEPO have been linked to tissue protection in the nervous system. In this study, we tested their effects in a model of neonatal hypoxia-ischemia (HI) in 7-day-old rats (unilateral carotid ligation and exposure to 7.7% O_2 for 50 min). EPO (10 U/g body weight 80 ng/g; *n* 24), asialoEPO (80 ng/g; *n* 23) or vehicle (phosphate-buffered saline with 0.1% human serum albumin; *n* 24) was injected intraperitoneally 4 h before HI. Both drugs were protective, as judged by measuring the infarct volumes, neuropathological score and gross morphological score. The infarct volumes were significantly reduced by both EPO (52%) and asialoEPO (55%) treatment, even though the plasma levels of asialoEPO had dropped below the detection limit (1 pM) at the onset of HI, while those of EPO were in the nanomolar range. Thus, a brief trigger by asialoEPO before the insult appears to be sufficient for protection. Proteomics analysis after asialoEPO treatment alone (no HI) revealed at least one differentially up-regulated protein, synaptosome-associated protein of 25 kDa (SNAP-25). Activation (phosphorylation) of ERK was significantly reduced in asialoEPO-treated animals after HI. EPO and the nonerythropoietic asialoEPO both provided significant and equal neuroprotection when administered 4 h prior to HI in 7-day-old rats. The protection might be related to reduced ERK activation and up-regulation of SNAP-25.

Keywords: asialoerythropoietin, erythropoietin, hypoxia, ischemia, neonatal, neuroprotection.

Hypoxic ischemic brain injury (HI) is one of the major causes of subsequent neurological, life long disability in both preterm and term infants. The injury develops over hours to days after the insult, and several mechanisms of injury have been identified; however, so far no treatment strategies have been found reliable in mitigating the neurological injury or resulting impairments (Hagberg *et al*. 2001). The neuropathology of brain injury after HI includes focal ischemic infarction, selective neuronal necrosis, inflammation and apoptosis (Hagberg 1992; Volpe 2001; Hagberg *et al*. 2002). During normal development more than half of the neurons are lost through apoptosis in certain brain regions (Raff *et al*. 1993). The immature brain has been suggested to retain this developmental cell death program to some extent, and apoptosis related mechanisms may play a more important role after HI in the immature than in the adult brain (Ni *et al*. 1998; Hu *et al*. 2000; Blomgren *et al*. 2001; Gill *et al*. 2002). Many key elements of apoptosis have been demonstrated to be activated and even up regulated in the immature

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Abbreviations used: asialoEPO, asialoerythropoietin; EPO, erythropoietin; EPOR, erythropoietin receptor; ERK, extracellular signal-related kinase; HI, hypoxia-ischemia; i.c.v., intracerebroventricular; i.p., intraperitoneal; i.v., intravenous; MAP2, microtube associated protein 2; PBS, phosphate-buffered saline; SNAP-25, synaptosomal-associated protein-25 kDa.

brain, such as caspase 3 (Cheng *et al*. 1998; Blomgren *et al*. 2001; Wang *et al*. 2001), AIF (Zhu *et al*. 2003), APAF 1 (Ota *et al*. 2002), Bcl 2 (Merry *et al*. 1994) and Bax (Vekrellis *et al*. 1997).

Human erythropoietin is a sialoglycoprotein (molecular weight *c*. 30 kDa) containing a 165 amino acid residue backbone (Jelkmann 1992). This cytokine has been associ ated mainly with the formation of new erythrocytes by protecting erythroid progenitors in the bone marrow against apoptosis, and the clinical applications of recombinant human EPO (rhEPO) have so far been focused mainly on the treatment of anemias. EPO mRNA was shown to be up regulated in several tissues after hypoxia, including the brain (Tan *et al*. 1992), and astrocytes have been suggested to mediate hypoxic preconditioning by producing EPO, thereby increasing the resistance of neurons to subsequent insults (Grimm *et al*. 2002; Ruscher *et al*. 2002). Both EPO and its receptor (EPOR) have been identified in the brain of several mammals (Digicaylioglu *et al*. 1995; Marti *et al*. 1996), including humans (Juul *et al*. 1999b). It appears that EPO acts on several different levels, such as attenuation of apoptosis (Juul *et al*. 1998; Digicaylioglu and Lipton 2001; Siren *et al*. 2001; Celik *et al*. 2002), excitotoxicity (Kawa kami *et al*. 2001), oxygen free radicals (Calapai *et al*. 2000; Digicaylioglu and Lipton 2001) and inflammation (Brines *et al*. 2000). Interestingly, despite its size, EPO did readily cross the blood brain barrier, reach hippocampal and cortical neurons (Erbayraktar *et al*. 2003), or mediate protection against focal ischemia in adult rats (Brines *et al*. 2000). The concentrations of EPO in plasma and CSF were higher in asphyxiated infants than in controls, but this was not the case after meningitis (Juul *et al*. 1999a), indicating that EPO was selectively increased in the CSF by hypoxia. Neuroprotective concentrations of rhEPO were detected in the cerebrospinal fluid after a single intravenous (i.v.) or intraperitoneal (i.p.) injection in fetal sheep and non human primates (Juul *et al*. 2004). In adult rodents, EPO treatment has provided neuroprotection when administered intracerebroventricular (i.c.v.) before (Bernaudin *et al*. 1999) and after (Sakanaka *et al*. 1998; Wen *et al*. 2002) the insult, but also systemic (i.p.) administration before (Siren *et al*. 2001) and after (Brines *et al*. 2000; Erbayraktar *et al*. 2003) an ischemic insult was effective. In all these settings, EPO was present at high plasma concentrations during the development of tissue damage. Recent reports indicate that EPO treatment is protective also in the neonatal setting, both after i.c.v. (Aydin *et al*. 2003) and i.p. administration in 7 day old rats (Kumral *et al*. 2003) and mice (Matsushita *et al*. 2003). The mech anisms mediating this protection *in vivo* have not been elucidated. EPO treatment is attractive because it has been in clinical use for years, and is also considered safe for pediatric purposes. However, multiple dosing may cause potentially harmful increases in hematocrit that augment brain injury (Wiessner *et al*. 2001). EPO devoid of sialic acid has been demonstrated to retain the neuroprotective properties of EPO without affecting hematocrit (Erbayraktar *et al*. 2003). It has been suggested that EPO or asialoEPO trigger neuroprotec tive signaling cascades that are memorized by cells. Due to its extremely short plasma half life, asialoEPO is an ideal tool for studies addressing the cellular changes triggered by EPO and relevant for delayed tissue protection, but this type of EPO derivative has not been tried in the immature brain. The present study was undertaken to compare the effects of EPO and the nonerythropoietic asialoEPO in a model of perinatal HI.

Materials and methods

Induction of hypoxia ischemia and drug administration

Unilateral hypoxia ischemia was induced in 7 day old Wistar rat pups (from Charles River, Sulzfeld, Germany) of either sex using the Rice Vannucci model (Rice *et al*. 1981). Animals were anesthetized with halothane (5% for induction, 1.5 3.5% for maintenance) in a mixture of nitrous oxide and oxygen (1 : 1), and the duration of anesthesia was < 5 min. The left common carotid artery was cut between double ligatures of prolene sutures (6 0). After the surgical procedure the wounds were infiltrated with a local anesthetic, and the pups were allowed to recover for 1 h. The litters were placed in a chamber perfused with a humidified gas mixture (7.7% oxygen in nitrogen) for 50 min. The temperature in the incubator, and the temperature of the water used to humidify the gas mixture, was kept at 36°C. After hypoxic exposure the pups were returned to their dams and allowed to recover for up to 5 days. Control pups were neither subjected to ligation nor hypoxia. Animals were injected i.p. with EPO (10 U/g body weight, equivalent to 80 ng/g) (Dragon Pharmaceuticals, Vancouver, Canada), asialoEPO (80 ng/g body weight), produced at Lundbeck as described earlier (Erbayraktar *et al*. 2003), or vehicle [phosphate buffered saline (PBS) with 0.1% human serum albumin] 4 h before the insult. In a separate series, animals were injected i.p. twice, at 24 h and 4 h before the insult, with EPO (5 U/g body weight, equivalent to 40 ng/g), asialoEPO (40 ng/g body weight) or vehicle (PBS with 0.1% human serum albumin). All animal experimentation was approved by the Ethical committee of Göteborg (204 2001 and 288 2002).

Assessment of brain damage

Infarct volume measurement

Five day post HI pups were deeply anesthetized and perfusion fixed with 5% formaldehyde in 0.1 M PBS. The brains were rapidly removed and immersion fixed in 5% formaldehyde at 4°C for 24 h. After dehydration with graded ethanol and xylene, the brains were paraffin embedded and cut into 5 μm frontal sections. Every 100th section was stained for microtubule associated protein 2 (MAP2). The areas in the cortex, striatum, thalamus and hypothalamus displaying loss of MAP2 staining were measured using Micro Image (Olympus, Tokyo, Japan) and the volumes calculated according to the Cavalieri Principle using the following formula: V $\Sigma A \times p \times T$, where V total volume, ΣA is the sum of the areas measured, p the inverse of the sections sampling fraction, and T

is the section thickness. The investigator measuring the MAP2 negative areas and calculating the volumes was blinded to the treatment of the animals. Total tissue loss was calculated by subtracting the MAP2 positive volume of the ipsilateral hemisphere from the contralateral hemisphere.

Neuropathology score

Brain injury in different regions was evaluated using a semiquantitative neuropathological scoring system as described previously (Hedtjärn *et al*. 2002). Briefly, sections were stained with thionin/acid fuchsin and scored by an observer blinded to the treatment of the animals. The cortical injury was graded from 0 to 4, 0 being no observable injury and 4 confluent infarction encompassing most of the hemisphere. The damage in hippocampus, striatum and thalamus was assessed both with respect to hypotrophy (shrinkage) (0 3) and observable cell injury/infarction (0 3) resulting in a neuropathological score for each brain region (0 6). The total score (0 22) was the sum for all four regions.

Gross morphology score

Gross morphology scoring was performed according to a method modified from Yager *et al*. (1992). After dissecting out the brain, the degree of injury was evaluated by inspection of the brain surface. Grade 0, normal, equal size of the two hemispheres and no visible white lesion; grade 1, a small white lesion plaque; grade 2, hypotrophy and large cysts in the ipsilateral hemisphere; grade 3, only parasaggital viable tissue left in the whole midline; and grade 4, total loss of the ipsilateral hemisphere.

CA1 neuronal count

The number of neurons in one visual field of CA1 5 days post HI was counted by an observer blinded to the treatment of the animals (400 × magnification, one visual field 0.196 mm^2). One section per animal was counted in the vehicle treated (n 24), EPO treated (n 24) and asialoEPO treated (n 23) animals and the average was compared between groups.

Pharmacokinetics

EPO or asialoEPO were administered i.p. (80 ng/g body weight) to 7 day old rats and blood was sampled at 4 min, 10 min, 1 h and 4 h (n 3 per time point) after the injection. Heparinized blood was centrifuged at 3200 × g for 10 min at 4°C and the plasma samples thus obtained were analyzed for EPO and asialoEPO using a validated ELISA at Lundbeck (Valby, Denmark), as described earlier (Erbayraktar *et al*. 2003).

Immunoblotting

Animals treated with asialoEPO or vehicle as above were killed by decapitation 3 h after HI (n 6 per time point). Littermates treated with asialoEPO but not subjected to HI were killed at the same time as those subjected to HI (7 h after the injection). The brains were rapidly dissected out on a bed of ice. The parietal cortex (including the hippocampus) and diencephalon (including the thalamus, hypothalamus and striatum) were snap frozen in liquid nitrogen and stored at −80°C. Tissue samples were homogenized by sonication in ice cold isolation buffer [15 mM Tris HCl, pH 7.6, 320 mM sucrose, 1 mM dithiothreitol, 1 mM $MgCl_2$, 3 mM EDTA K, 0.5% protease inhibitor cocktail (P8340; Sigma) and 50 mg/mL cyclosporine A], aliquoted and stored at −80°C. The protein concentration was determined according to Whitaker and Granum (1980), adapted for micro plates. Samples were mixed with an equal volume of concentrated (3 ×) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) buffer and heated (96°C) for 5 min. Individual samples were run on 4 20% Tris glycine gels (Novex, San Diego, CA, USA) and transferred to reinforced nitrocellulose (Schleicher & Schuell, Dassel, Germany) membranes. After blocking with 30 mM Tris HCl (pH 7.5), 100 mM NaCl and 0.1% Tween 20 (TBS T) containing 5% fat free milk powder for 1 h at room temperature, the membranes were incubated with primary antibodies: anti p ERK (#9101, 1 : 1000, Cell Signaling Technology, Inc. Beverly, MA. USA), anti ERK (#9102, 1 : 1000, Cell Signaling), anti SNAP 25 (sc 7538, 1 : 500, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), anti nitrotyrosine (1 μg/mL, HM12, Biomol, Plymouth Meeting, PA, USA), anti EPOR (M 20, sc 697, Santa Cruz Biotechnology, Santa Cruz, USA and MAB307, R & D Systems, Minneapolis, MN, USA) or anti actin (A2066, 1 : 200, Sigma, Stockholm, Sweden) at room temperature for 1 h followed by an appropriate peroxidase labeled, secondary antibody for 30 min at room temperature (horse anti mouse 1 : 2000, horse anti goat 1: 2000, or goat anti rabbit, 1 : 500, Vector, Burlingame, CA, USA). Immunoreactive species were visualized using the Super Signal Western Dura substrate (Pierce, Rockford, IL, USA) and a LAS 1000 cooled CCD camera (Fujifilm, Tokyo, Japan). Immunoreactive bands were quantified using the Image Gauge software (Fujifilm, Tokyo, Japan). Every sample was quantified 1 3 times on different membranes, and the average value was used as n 1.

Immunohistochemistry

Antigen recovery was performed by heating the sections in 10 mM boiling sodium citrate buffer (pH 6.0) for 10 min. Nonspecific binding was blocked for 30 min with 4% horse serum in PBS. Anti MAP2 (clone HM 2, 1 : 2000; Sigma) incubated for 1 h at room temperature, followed by a biotinylated horse anti mouse secondary antibody for 1 h (1 : 200, Vector, Burlingame, CA, USA). Endogenous peroxidase activity was blocked with 3% H_2O_2 for 5 min. Visualization was performed using Vectastain ABC Elite with 0.5 mg/mL 3,3′ diaminobenzidine (DAB) enhanced with 15 mg/mL ammonium nickel sulfate, 2 mg/mL beta D glucose, 0.4 mg/mL ammonium chloride and 0.01 mg/mL beta glucose oxidase (Sigma).

Proteomics analysis

The proteomics analysis was performed at ProteoSys AG (Mainz, Germany). Seven day old Wistar rats of either sex were injected i.p. with vehicle or asialoEPO (80 ng/g body weight) (n 24 per group). Animals were randomized to proteomics analysis or HI. Half of the animals (n 12 per group) were subjected to HI, killed 5 days later and evaluated by gross morphological scoring to verify the protective effect of asialoEPO. The other half of the animals (n 12 per group) were killed 4 h after the injection and the parietal cortex (including the hippocampus) was dissected out on ice from each hemisphere, snap frozen in liquid nitrogen and stored at −80°C. Triplicate samples of the total proteome and the phosphoproteome were analyzed. Briefly, the brains from the same group (vehicle or asialoEPO) were combined and pulverized under liquid nitrogen. For total proteome analysis about 100 mg of ground tissue

was homogenized in 1 mL alkylation buffer (0.1 M Tris (pH 8.8), 2% (SDS). For phosphoproteome enrichment, about 1 g of ground tissue was extracted in extraction buffer (50 mM MOPS (pH 6.8), 4% Zwittergent 3 12, 2% Triton X 100, 5 mM NaF, 5 mM sodium glycerophosphate, 1 mM activated sodium orthovanadate, 5 mM Na EDTA, 1 × Complete® Protease Inhibitor) yielding about 60 mg of protein. Ni NTA columns were activated by washing four times with two bed volumes regeneration buffer (2% Trition X 100, 50 mM Tris HCl (pH 7.4), 0.5 M NaCl, 0.1 M ETDA Na_4), followed by eight bed volumes MilliQ water and four bed volumes of activation buffer (100 mM $FeCl_3$). After activation the column was washed with eight bed volumes MilliQ water and eight bed volumes binding buffer (50 mM bis (hydroxyethyl)piperazine (pH 3.4), 4% zwittergent 3 10, 2% Triton X 100). Protein solutions after DNA removal were submitted to a gel filtration column (NAP 10 or NAP 25) to exchange the buffer against the binding buffer [50 mM bis (hydroxyethyl)piperazine (pH 3.4), 4% Zwittergent 3 10, 2% Triton X 100]. The extract was subsequently loaded onto an activated Fe NTA Agarose column (maximum 2 mg per 0.5 mL settled bed volume). The column was then washed three times with two bed volumes of binding buffer and eluted with two bed volumes elution buffer (50 mM bis (hydroxyethyl)piperazine (pH 3,4), 4% Zwittergent 3 10, 2% Triton X 100, 50 mM NaH_2PO_4). Flow through, wash and elution fractions were collected. The eluate was TCA precipitated, the pellet appropriately washed and recovered in alkylation buffer (0.1 M Tris (pH 8.8), 2% (SDS). Alkylation and sample treatment were performed exactly as described (Vuong *et al*. 2000; Cahill *et al*. 2003).

The differential and quantitative protein expression analysis was performed using the ProteoTope method (Cahill *et al*. 2003) which is based on radioiodination, 2D PAGE and high sensitivity radio imaging. In brief, small amounts of each sample were labeled with ^{125}I and ^{131}I for differential pattern control. The signals from these two isotopes were distinguished in one 2D PAGE gel to generate a quantitative multicolor differential display of proteins. A direct comparison of integrated spot intensities for the samples run on one gel was used for further analysis. In parallel, silver stained gels were produced for spot picking and protein identification and for complementary quantification. In general, a spot was selected for further analysis if the *t* test probability was higher than 95% and the expression ratio higher than 1.5. At that point the image quality was checked to assure that the spot was consistently detected on all images. Protein identification was based on different mass spectr ometric methods. Briefly, gel plugs of selected protein spots were excised and the proteins contained in the gel plugs digested using trypsin. The resulting solution was analyzed first with a high throughput peptide mass fingerprint procedure based on MALDI TOF MS. For those spots where no unambiguous identification was achieved a fragment ion analysis based on LC ESI IonTrap MS/MS was added (Cahill *et al*. 2003). For the identification of the proteins the peptide masses extracted from the mass spectra were searched against the NCBI non redundant protein database (http://www.ncbi.nlm.nih.gov) using MASCOT software version 1.9 (Matrix Science, London).

Statistics

ANOVA followed by Fischer's PLSD *post hoc* test was used for comparing the results from infarct volume measurements, gross morphology score, neuropathology score, CA1 neuron counting, and p ERK and SNAP 25 immunoblot quantification. Simple linear regression analysis was used for the comparison of gross morphol ogy score, infarct volume measurement, total tissue loss and neuropathology score. Unpaired *t* test was used to assess statistical significance of the differences between the spot intensities on control and sample gel in proteomics analysis.

Results

Evaluation of brain damage

Brain damage was evaluated using three independent methods, infarct volume measurement, neuropathology score and gross morphology score. The total infarct volume (mean ± SEM) in the ipsilateral hemisphere in vehicle treated rats was 31.5 ± 6.3 mm^3 ($n = 24$), 15.0 ± 2.9 mm^3 in EPO treated rats ($n = 24$) (51.7% reduction, $p = 0.0146$), and 13.5 ± 4.1 mm^3 in asialoEPO treated rats ($n = 23$) (54.7% reduction, $p = 0.0085$) (Fig. 1a,b). There was considerable variation in different brain regions. The changes were most pronounced in the cortex (53.7% reduction in EPO treated animals and 56.2% in asialoEPO treated animals, $p = 0.0136$ and 0.0083, respectively) and least in thalamus (14.9% in EPO treated animals and 28.6% in asialoEPO treated animals, non significant) (Fig. 1c). The protective effect was confirmed using gross morphology scoring (Fig. 2a) and neuropathology score (Table 1). Interestingly, when the drugs were adminis tered twice, at 24 h and 4 h prior to the insult (5 U/g or 40 ng/g each, i.e. the same total dose of 10 U/g or 80 ng/g), no protection was observed as judged by gross morphology score (Fig. 2b). The presence or absence of human serum albumin in the vehicle did not make any difference in this respect (data not shown).

In histological sections, the CA1 region of vehicle treated rats displayed a marked decline in viable neurons when compared with the CA1 of EPO or asialoEPO treated rats (Fig. 3a). The number of neurons per visual field per section in the CA1 was 19 ± 3 (mean ± SEM) in vehicle treated rats compared to 25 ± 2 in EPO treated and 30 ± 3 for asialo EPO treated rats, i.e. a 50% increase in the asialoEPO treated animals, $p = 0.0036$ (Fig. 3b).

Pharmacokinetics

As neonatal animals may differ significantly from adult rodents we repeated pharmacokinetic studies for the relevant time span. The plasma concentration profiles of EPO and asialoEPO during the first four hours after an i.p. injection were strikingly similar to those observed in adults (Erbay raktar *et al*. 2003) (Fig. 4). Basal EPO concentrations were < 1 pM, i.e. below the detection limit. After injection, the plasma EPO concentrations increased from 50 to 90 pM at 4 10 min to values of about 2000 pM after 60 240 min.

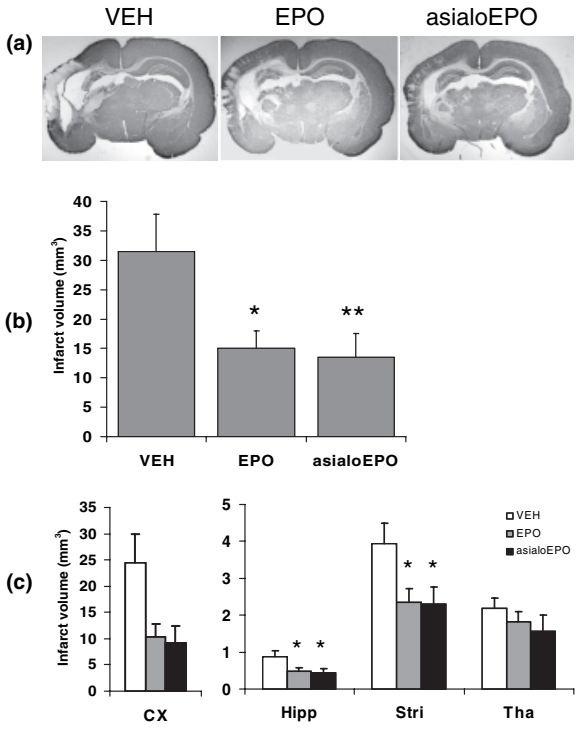

Fig. 1 Infarct volumes after EPO and asialoEPO treatment. The infarct volumes (MAP2 negative volumes) were quantified 5 days post HI. (a) Representative pictures of MAP2 staining from animals treated with vehicle (VEH), erythropoietin (EPO) or asialoerythro poietin (asialoEPO). (b) The average total infarct volume ± SEM is indicated for the vehicle ($n = 24$), EPO ($n = 24$) and asialoEPO treated ($n = 23$) animals. (c) The regional differences are depicted, showing the average infarct volumes ± SEM in the cortex (CX), hippocampus (Hipp), striatum (Stri) and thalamus (Tha). *$p < 0.05$, **$p < 0.01$, using ANOVA and Fischer's *post hoc* test.

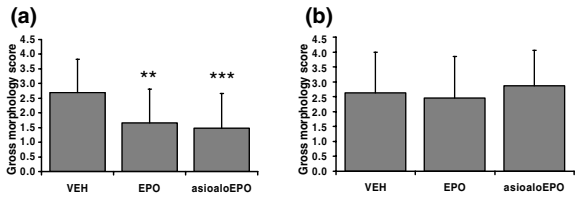

Fig. 2 Assessment of brain injury using gross morphology score after EPO and asialoEPO treatment. Gross morphology scoring was per formed 5 days post HI. (a) Treatment with vehicle (VEH) ($n = 24$), EPO ($n = 24$), and asialoEPO ($n = 23$) once 4 h prior to HI. Dose: 80 ng/g body weight for EPO and asialoEPO. (b) Treatment with vehicle (VEH) ($n = 31$), EPO ($n = 29$) and asialoEPO ($n = 32$) twice at 24 and 4 h prior to HI. Dose: 40 ng/g body weight per injection for EPO and asialoEPO. **$p < 0.01$, ***$p < 0.001$, using ANOVA and Fischer's *post hoc* test.

Also asialoEPO was above the detection limit already after 4 min, rose to a plateau of about 50 pM between 10 and 60 min and dropped below the detection limit at 4 h (Fig. 4), the time point when HI was performed. Thus, effects of

Table 1 Assessment of brain injury using neuropathology score after EPO and asialoEPO treatment

Brain regions	VEH	EPO	*p*	AsialoEPO	*p*
Cortex	2.70 ± 1.08	1.88 ± 0.95	0.075	1.73 ± 0.88	0.001
Hippocampus	3.74 ± 1.52	2.89 ± 1.12	0.032	2.79 ± 0.84	0.011
Striatum	3.55 ± 1.37	2.44 ± 0.75	0.001	2.46 ± 0.92	0.002
Thalamus	2.99 ± 1.32	2.27 ± 0.53	0.016	1.93 ± 1.13	0.005
Total score	12.99 ± 4.89	9.48 ± 2.69	0.003	8.91 ± 3.10	0.001

Neuropathology scores for the different brain regions indicated, as well as the total score, in animals treated with vehicle (VEH) ($n = 23$), EPO ($n = 24$) or asialoEPO ($n = 24$).

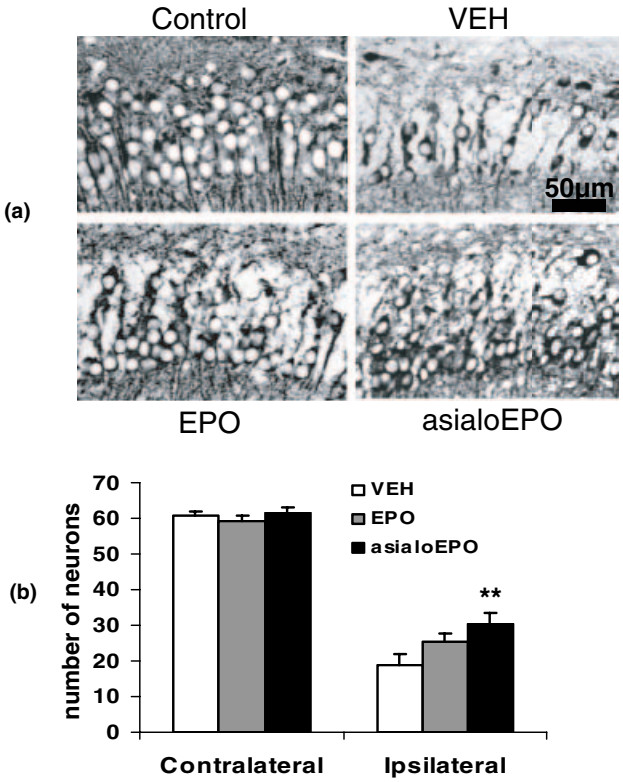

Fig. 3 Neuronal loss in the CA1. (a) Representative MAP2 stainings of the CA1 area 5 days after the insult in control animals not subjected to HI (control) and animals treated with vehicle (VEH), erythropoietin (EPO), or asialoerythropoietin (asialoEPO). The black or dark MAP2 staining is apparent in the cytosol of CA1 neurons, whereas the nuclei remain unstained and virtually white. (b) The average total number of neurons in the CA1 ± SEM in the contralateral and ipsilateral hemi spheres in the three groups. *$p < 0.05$, **$p < 0.01$, using ANOVA and Fischer's *post hoc* test.

asialoEPO cannot be attributed to the opening of the blood brain barrier during HI, and any signal observed at 4 h in brain was likely triggered earlier by asialoEPO and memor ized. The ventricular system in neonatal rat brains is too small to allow sampling of cerebrospinal fluid, so we attempted analyzing brain tissue extracts at the same time

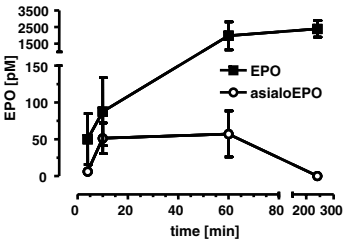

Fig. 4 The plasma concentrations of EPO and asialoEPO after a single injection. EPO or asialoEPO were injected i.p. at a dose of 80 ng/g. The concentrations in blood plasma were measured over a 4 h period, sampled from three animals per data point. Data are dis played as means ± SD.

points as the plasma measurements. The results merely reflected the plasma concentrations, but at a much lower level, in the case of asialoEPO even close to detection level because of different immunoreactivity (data not shown). This makes major accumulation of EPO or asialoEPO in the brain unlikely.

Proteomics analysis

We compared total protein extracts from asialoEPO treated versus vehicle treated cortices 4 h after injection, the time point when HI would have been induced. Based on our selection criteria of a significant ($p < 0.05$) up regulation of > 50%, three differential protein spots were identified on 2D gel patterns. However, these proteins could not be identified by mass spectrometry. One identified protein, NADH dehydrog enase (ubiquinone) Fe S protein 1, was significantly down regulated. Other proteins remained entirely unaffected. Phos phoprotein enrichment focuses the molecular analysis on a functionally important negatively charged subset of approxi mately 15% of total proteins. Quantification by radioactive differential display revealed one significantly up regulated protein when comparing samples from animals treated with asialoEPO versus control. The protein, later identified by MALDI MS as synaptosome associated protein of 25 kDa (SNAP 25), was low abundant in the phosphoproteome, but was identified both in silver stained (Fig. 5) and in radioactive quantitative gels ($p < 0.01$). SNAP 25 displayed a 50 60% up regulation using either method. Three further protein spots were significantly increased, but could not be identified. The bulk of other proteins remained unaffected.

We used immunoblotting to check for SNAP 25 up regulation, but we were not able to detect changes in the overall levels of SNAP 25. Animals treated with EPO or asialoEPO alone (no HI) did not display significant differences in the levels of SNAP 25 4 h after the injection, neither in homogenates, nor in a synaptosomal fraction (where SNAP 25 would conceivably be enriched; data not shown). Animals treated with asialoEPO and 4 h later subjected to HI did not display significant differences in the levels of SNAP 25 3 or 24 h (not shown) after the insult. SNAP 25 was up regulated in the phosphoproteome, not in the total proteome, but there was no antibody available specific for phosphorylated SNAP 25. This presumably explains the negative Western blotting data.

Signal transduction mechanisms

A number of signal transduction proteins previously implied in EPO signaling were investigated using immunoblotting and immunohistochemistry. The levels of phosphorylated Akt (p AKT), extracellular signal related kinase (p ERK), GSK 3 beta (p GSK 3 beta), nitrotyrosine, as well as X linked inhibitor of apoptosis protein (XIAP) and EPO receptor (EPOR) were measured using immunoblotting 4 h after EPO or asialoEPO treatment alone (no HI), the time point when HI would have been induced. None of these proteins were found to be differentially regulated after treatment (data not shown). However, when asialoEPO treatment was combined with HI, a significant difference could be detected in the levels of p ERK (Fig. 6). As described earlier, the levels of p ERK increased after HI (Wang *et al*. 2003), but in the asialoEPO treated animals this increase was significantly reduced by more than 30% when measured 3 h after HI (7 h after treatment), compared with vehicle treated rats (Fig. 6). The tendency was the same for the basal levels of p ERK (without HI), but the difference was not significant (Fig. 6). Total ERK (Fig. 6), as well as the other signal transduction proteins investigated, p AKT, p GSK 3 beta, nitrotyrosine, XIAP and EPOR, were not differentially regulated after asialoEPO combined with HI using western blotting and/or immunohistochemistry (data not shown).

Discussion

Tissue protection

Neuroprotection using different EPO treatment regimes has been demonstrated in both the adult (Sadamoto *et al*. 1998; Bernaudin *et al*. 1999; Brines *et al*. 2000; Siren *et al*. 2001; Wen *et al*. 2002; Erbayraktar *et al*. 2003) and neonatal (Aydin *et al*. 2003; Kumral *et al*. 2003; Matsushita *et al*. 2003; Sun *et al*. 2004) brain. We found that both EPO and the nonerythropoietic asialoEPO provided neuroprotection in a model of neonatal HI when administered as a single, i.p. injection 4 h prior to HI. The protection was confirmed using three independent methods of brain damage assessment. This is the first report demonstrating that the nonerythropoietic asialoEPO protects against neonatal HI brain damage. The dosage and timing appear to be critical when using EPO and its derivatives. Even though significant protection was observed using 80 ng/g asialoEPO or using 10 U/g EPO (yielding 40 times higher plasma concentrations than asialo EPO) 4 h prior to HI, the same total dose of EPO or asialoEPO (10 U/g or 80 ng/g) was ineffective when administered using

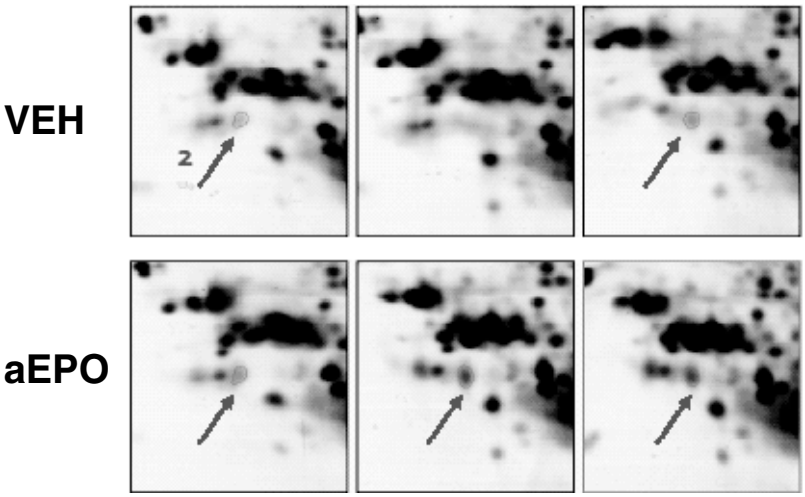

Fig. 5 Identification and differential regulation of SNAP 25 in the phosphoproteome after asialoEPO administration. Silver stained gels showing the SNAP 25 spots. These six figures show triplicate silver stained gels from vehicle (VEH) and asialoEPO (aEPO) treated animals, with detailed views of the SNAP 25 protein spot (arrow). The spot was subsequently identified using MALDI.

two injections at 24 h and 4 h prior to HI (5 U/g or 40 ng/g on each occasion). This was repeated twice, using two different types of vehicle (with or without human serum albumin), but the results were identical (data not shown). This was probably not due to an insufficient dose, because 5 U/g is a commonly used dose in other paradigms, including neonatal HI (Matsushita *et al*. 2003). It is conceivable that the 24 h pretreatment in our model may have down regulated the EPO receptor or other effector mechanisms so that the effect of the second dose was diminished.

When systematically comparing the three independent methods of assessing brain damage, infarct volume measurement (and the related total tissue loss), gross morphology score, and neuropathological score using simple linear regression analysis, there was a significant positive linear correlation between all these three (or four) evaluation methods (Fig. 7). This strongly suggests that gross morphology score, which is the simplest and quickest way to assess brain injury, can be used at least for the initial evaluation of possible protective effects.

Pharmacokinetics

The rapid clearance of asialoEPO from the circulation is the reason why it does not affect the erythropoiesis (Erbayraktar *et al*. 2003). Even though asialoEPO was no longer detectable in plasma at the onset of HI, it still provided the same, or an even better (in the case of CA1 neuronal counts) degree of protection. This indicates that the protective mechanisms were activated and remained for a period of time, similar to the concept of preconditioning. Also, it has been demonstrated that even a short lasting exposure (5 min) to EPO was sufficient to increase the resistance of neurons to glutamate toxicity *in vitro* (Morishita *et al*. 1997). In clinical settings, it may be an advantage that asialoEPO does not stimulate the erythropoiesis, particularly in a situation where multiple dosing is required. An increased number of red blood cells may aggravate brain injury (Wiessner *et al*. 2001) or stimulate the formation of hyperactive platelets (Wolf *et al*. 1997a) and predispose to thrombosis (Wolf *et al*. 1997b), effects to be avoided when treating an asphyxiated infant or a stroke patient.

Proteomics

A proteomics approach was undertaken to detect differentially regulated proteins in the total proteome as well as the phosphoproteome of the cerebral cortex 4 h after a single injection of asialoEPO. There were three significantly increased protein spots matching the selection criteria in the total proteome and four in the phosphoproteome, but only one of these could be identified, SNAP 25 in the phosphoproteome. In general, the concentration changes detected were subtle and most proteins remained unaffected. It is possible that the changes observed were diluted by unrelated cell populations or compartments. SNAP 25 was quite low abundant in the phosphoproteome, but was consistently up regulated as judged by two independent methods, silver stain and ProteoTope gels. SNAP 25 is a neuronal, soluble *N* ethylmaleimide sensitive factor attachment protein receptor (SNARE) syntaxin involved in vesicle trafficking. It belongs to a family of evolutionarily conserved proteins whose members are essential for exocytosis and has been exclusively detected in neuronal tissues (Bark *et al*. 1995). It has been reported that SNAP 23, an isoform of SNAP 25, was induced 2 h after stimulation by various cytokines, including EPO, in the erythroid cell line SKT6 (Morikawa *et al*. 1998). The functional role of SNAP 25 in asialoEPO mediated neuroprotection, if any, might be related to synaptic transmission. The results from the phosphoproteome could not be confirmed on immunoblots, neither following EPO or asialoEPO treatment alone, nor after asialoEPO followed by

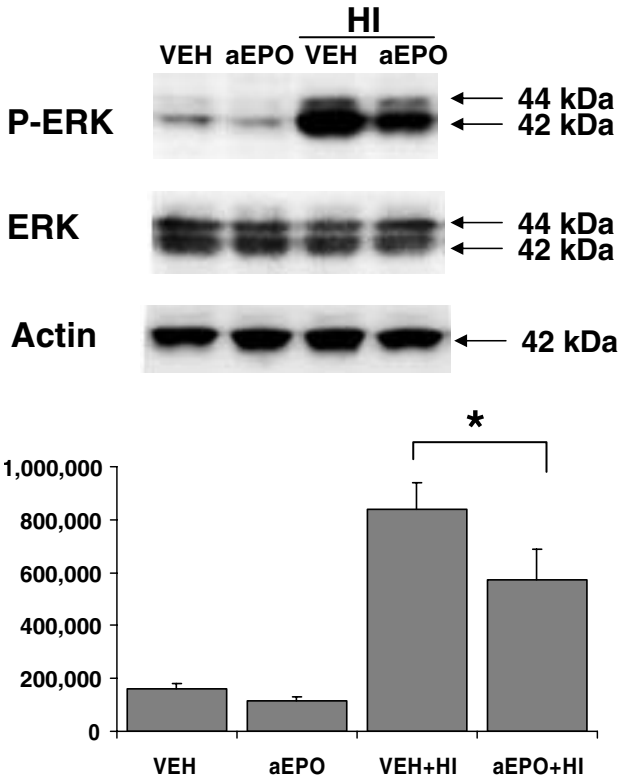

Fig. 6 The immunoreactivity of activated ERK was decreased in asi aloEPO treated animals after hypoxia ischemia. All rats were injected i.p. with either asialoEPO (aEPO) or vehicle 4 h before HI and killed 3 h after the insult. Rats not subjected to HI were killed at the same time, i.e. 7 h after the injection. The upper panel shows representative immunoblots of phosphorylated ERK (p ERK), total ERK (ERK) and actin (as a control for equal loading), demonstrating low levels of p ERK in control animals, and a substantial increase of p ERK immunoreactivity in the ipsilateral hemisphere 3 h after HI, less pro nounced after asialoEPO treatment. The lower panel shows the average p ERK immunoreactivity ± SEM after densitometric quantifi cation of individual samples ($n = 6$ in each group). *$p < 0.05$ using Student's *t* test.

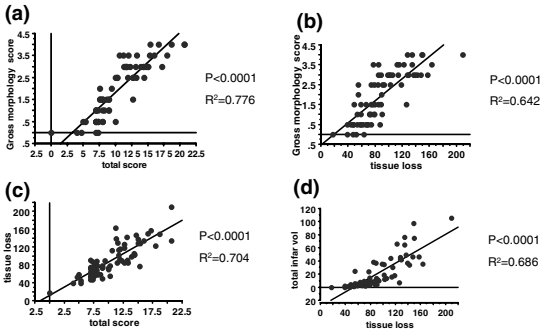

Fig. 7 Correlation between different methods for assessment of brain damage. Simple linear regression analysis showed that the gross morphology score, neuropathology score, infarct volume and total tissue loss all show a positive, significant correlation. (a) Correlation between the gross morphology score and neuropathology score (total score), $R^2 = 0.776$. (b) Correlation between the gross morphology score and total tissue loss, R2 = 0.642. (c) Correlation between the total tissue loss and neuropathology score, $R^2 = 0.704$. (d) Correla tion between the total infarct volume and total tissue loss, $R^2 = 0.686$. The *p* value was < 0.0001 in all four analyses.

HI and evaluated either 3 or 24 h after the insult. The reason for this is probably that the antibody used detected total SNAP 25, while the phosphoproteome analysis detected changes in the phosphorylated form of SNAP 25, or that multiple isoforms exist. To elucidate this, a phospho specific antibody against SNAP 25 would be required. A recent study showed that PKA induced phosphorylation of SNAP 25 regulated the size of the releasable vesicle pool (Nagy *et al*. 2004).

Signaling mechanisms

Several mechanisms mediating the protective effects of EPO have been suggested: decreased glutamate toxicity (Morishita *et al*. 1997; Kawakami *et al*. 2001); neuronal anti apoptotic mechanisms (Juul *et al*. 1998; Renzi *et al*. 2002); reduced inflammation (Brines *et al*. 2000); up regulation of HSP 27 (Sun *et al*. 2004); decreased nitric oxide mediated injury (Digicaylioglu and Lipton 2001); direct antioxidant effects and indirect effects on endothelial cell growth (Hayashi *et al*. 1998). (For current reviews, see Juul 2000; Marti *et al*. 2000; Buemi *et al*. 2002; Chong *et al*. 2002). An interesting feature of EPO signaling is that it may act as a preconditioning factor, i.e. change a cell's sensitivity towards injury without being continuously present. This is difficult to examine using a molecule with a half life of several hours, but such experimental designs are possible using asialoEPO, which has a half life of merely a few minutes in plasma. Even though p AKT, p GSK 3 beta, p ERK, NO signaling, XIAP and EPOR have all been implicated in the signal transduction mechanisms mediating EPO protection, we were unable to detect any changes in the levels of these proteins (or NO mediated formation of nitrotyrosine) using immunoblotting or immunohistochemistry 4 h after a single injection. This may indicate that they are not involved in this paradigm; alternatively the changes may have been too small to be detected using immunoblotting of samples from the entire cortex, or the protective mechanisms do not require changes in the total levels to be effective. It is possible that the priming effects of an insult, such as hypoxia or HI, are required for the mechanisms to be activated. This is supported by studies where hypoxia induced EPOR expres sion in cultured neuronal cells (Chin *et al*. 2000; Yu *et al*. 2002). When we combined asialoEPO treatment with HI, we did find significantly decreased levels of p ERK 3 h post insult, but no effects on p AKT, p GSK 3 beta, XIAP, nitrotyrosine or EPOR at 3 or 24 h after HI. Previous studies

have described an increase in EPOR expression upon EPO treatment (Ruscher *et al*. 2002; Sun *et al*. 2004), and asialoEPO has been shown to bind to EPOR with a similar affinity as EPO (Erbayraktar *et al*. 2003), but we were unable to find any changes of EPOR under any conditions. Sun *et al*. (2004) found enhanced EPOR expression 24, 72 h and 7 days after (repeated) EPO treatment, while we sampled brain tissue 4 h after the treatment, when HI would have been induced.

The effect on p ERK is consistent with our previous findings, where p ERK was detected in injured neurons for at least 8 h after HI, as judged by double labeling with markers of injury (Wang *et al*. 2003). Involvement of ERK in EPO mediated signaling has also been demonstrated by others (Shan *et al*. 1999; Siren *et al*. 2001; Mori *et al*. 2003). Surprisingly, we did not find any effect on the levels of caspase 3 , 8 or 9 like activity, measured using fluorogenic peptide substrates 24 h after the insult (data not shown), the time point when caspase activities peak in this model (Blomgren *et al*. 2001; Wang *et al*. 2001; Zhu *et al*. 2003), despite the fact that we observed tissue protection. We expected the extent of tissue damage and caspase activity to show a positive correlation, regardless of whether there is a causal or indirect relation between them. Possibly, non caspase dependent types of cell death (Leist and Jäättelä 2001) may play a major role in this model (Zhu *et al*. 2003), and EPO might affect tissue protection more via these pathways than through direct caspase inhibition. Another issue related to insult priming is the permeability of the blood brain barrier (BBB) to EPO and asialoEPO. On the one hand there are reports showing that EPO administered to preterm and term infants (Juul *et al*. 1997) or normal neonatal rats (Dame *et al*. 2001) did not lead to elevated levels in the CSF in the absence of brain injury. On the other hand there are reports showing that a single EPO dose of 5 U/g i.p. did lead to increased concentrations in the CSF in adult rodents (Brines 2002) as well as in neonatal sheep, non human primates (Juul *et al*. 2004), and humans (Ehrenreich *et al*. 2004). Furthermore, tissue sec tions obtained 4 h after i.v. administration of radio iodinat ed asialoEPO displayed labeling in hippocampal and cortical neurons, cells which also express EPOR in the normal adult rat brain (Erbayraktar *et al*. 2003). Further more, the fact that we find tissue protection after asialoEPO treatment, which is no longer present at time points when the BBB may be opened by the HI damage, supports the hypothesis that EPO protection is mediated through a priming mechanism and that it may pass the BBB without prior permeabilization.

In summary, EPO and the nonerythropoietic asialoEPO both provided significant neuroprotection when administered 4 h prior to hypoxia ischemia in 7 day old rats. The protective effect might be related to a reduction of ERK activation and SNAP 25 up regulation. Further work is needed to further elucidate the signaling mechanisms and the optimal treatment regimes, but it is clear that these drugs, particularly asialoEPO, may provide powerful and safe tools in the management of asphyxiated neonates to treat and prevent brain injury.

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References

Aydin A., Genc K., Akhisaroglu M., Yorukoglu K., Gokmen N. and Gonullu E. (2003) Erythropoietin exerts neuroprotective effect in neonatal rat model of hypoxic-ischemic brain injury. *Brain Dev.* **25**, 494 498.

Bark I. C., Hahn K. M., Ryabinin A. E. and Wilson M. C. (1995) Differential expression of SNAP-25 protein isoforms during divergent vesicle fusion events of neural development. *Proc. Natl Acad. Sci. USA* **92**, 1510 1514.

Bernaudin M., Marti H. H., Roussel S., Divoux D., Nouvelot A., MacKenzie E. T. and Petit E. (1999) A potential role for erythro poietin in focal permanent cerebral ischemia in mice. *J. Cereb Blood Flow Metab.* **19**, 643 651.

Blomgren K., Zhu C., Wang X., Karlsson J. O., Leverin A. L., Bahr B. A., Mallard C. and Hagberg H. (2001) Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia-ischemia: a mech anism of 'pathological apoptosis'? *J. Biol. Chem.* **276**, 10 191 10 198.

Brines M. (2002) What evidence supports use of erythropoietin as a novel neurotherapeutic? *Oncology (Huntingt.)* **16**, 79 89.

Brines M. L., Ghezzi P., Keenan S., Agnello D., de Lanerolle N. C., Cerami C., Itri L. M. and Cerami A. (2000) Erythropoietin crosses the blood brain barrier to protect against experimental brain injury. *Proc. Natl Acad. Sci. USA* **97**, 10 526 10 531.

Buemi M., Aloisi C., Cavallaro E., Corica F., Floccari F., Grasso G., Lasco A., Pettinato G., Ruello A., Sturiale A. and Frisina N. (2002) Recombinant human erythropoietin (rHuEPO): more than just the correction of uremic anemia. *J. Nephrol.* **15**, 97 103.

Cahill M. A., Wozny W., Schwall G. *et al.* (2003) Analysis of relative isotopologue abundances for quantitative profiling of complex protein mixtures labelled with the acrylamide/D3-acrylamide al kylation tag system. *Rapid Commun. Mass Spectrom.* **17**, 1283 1290.

Calapai G., Marciano M. C., Corica F., Allegra A., Parisi A., Frisina N., Caputi A. P. and Buemi M. (2000) Erythropoietin protects against brain ischemic injury by inhibition of nitric oxide formation. *Eur. J. Pharmacol.* **401**, 349 356.

Celik M., Gokmen N., Erbayraktar S., Akhisaroglu M., Konakc S., Ulukus C., Genc S., Genc K., Sagiroglu E., Cerami A. and Brines M. (2002) Erythropoietin prevents motor neuron apoptosis and

neurologic disability in experimental spinal cord ischemic injury. *Proc. Natl Acad. Sci. USA* **99**, 2258 2263.

Cheng Y., Deshmukh M., D'Costa A., Demaro J. A., Gidday J. M., Shah A., Sun Y., Jacquin M. F., Johnson E. M. and Holtzman D. M. (1998) Caspase inhibitor affords neuroprotection with delayed administration in a rat model of neonatal hypoxic-ischemic brain injury. *J. Clin. Invest.* **101**, 1992 1999.

Chin K. YuX., Beleslin-Cokic B., Liu C., Shen K., Mohrenweiser H. W. and Noguchi C. T. (2000) Production and processing of erythropoietin receptor transcripts in brain. *Brain Res. Mol. Brain Res.* **81**, 29 42.

Chong Z. Z., Kang J. Q. and Maiese K. (2002) Hematopoietic factor erythropoietin fosters neuroprotection through novel signal transduction cascades. *J. Cereb. Blood Flow Metab.* **22**, 503 514.

Dame C., Juul S. E. and Christensen R. D. (2001) The biology of erythropoietin in the central nervous system and its neurotrophic and neuroprotective potential. *Biol. Neonate* **79**, 228 235.

Digicaylioglu M. and Lipton S. A. (2001) Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades. *Nature* **412**, 641 647.

Digicaylioglu M., Bichet S., Marti H. H., Wenger R. H., Rivas L. A., Bauer C. and Gassmann M. (1995) Localization of specific erythropoietin binding sites in defined areas of the mouse brain. *Proc. Natl Acad. Sci. USA* **92**, 3717 3720.

Ehrenreich H., Degner D., Meller J. *et al.* (2004) Erythropoietin: a candidate compound for neuroprotection in schizophrenia. *Mol. Psychiatry* **9**, 42 54.

Erbayraktar S., Grasso G., Sfacteria A. *et al.* (2003) Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity *in vivo*. *Proc. Natl Acad. Sci. USA* **100**, 6741 6746.

Gill R., Soriano M., Blomgren K. *et al.* (2002) Role of caspase-3 activation in cerebral ischemia-induced neurodegeneration in adult and neonatal brain. *J. Cereb. Blood Flow Metab.* **22**, 420 430.

Grimm C., Wenzel A., Groszer M., Mayser H., Seeliger M., Samardzija M., Bauer C., Gassmann M. and Reme C. E. (2002) HIF-1-induced erythropoietin in the hypoxic retina protects against light-induced retinal degeneration. *Nat. Med.* **8**, 718 724.

Hagberg H. (1992) Hypoxic-ischemic damage in the neonatal brain: excitatory amino acids. *Dev. Pharmacol. Ther.* **18**, 139 144.

Hagberg H., Mallard C. and Blomgren K. (2001) Neuroprotection of the fetal and neonatal brain, in *Fetal and Neonatal Neurology and Neurosurgery*, 3rd edn. (Levene, M. I. Chervenak, F. A. and Whittle, M. J., eds), pp. 505 520. Churchill Livingstone, London.

Hagberg H., Ichord R., Palmer C., Yager J. Y. and Vannucci S. J. (2002) Animal models of developmental brain injury: relevance to human disease. A summary of the panel discussion from the Third Hershey Conference on Developmental Cerebral Blood Flow and Metabolism. *Dev. Neurosci.* **24**, 364 366.

Hayashi T., Abe K. and Itoyama Y. (1998) Reduction of ischemic damage by application of vascular endothelial growth factor in rat brain after transient ischemia. *J. Cereb. Blood Flow Metab.* **18**, 887 895.

Hedtjärn M., Leverin A. L., Eriksson K., Blomgren K., Mallard C. and Hagberg H. (2002) Interleukin-18 involvement in hypoxic-ischemic brain injury. *J. Neurosci.* **22**, 5910 5919.

Hu B. R., Liu C. L., Ouyang Y., Blomgren K. and Siesjo B. K. (2000) Involvement of caspase-3 in cell death after hypoxia-ischemia declines during brain maturation. *J. Cereb. Blood Flow Metab.* **20**, 1294 1300.

Jelkmann W. (1992) Erythropoietin: structure, control of production, and function. *Physiol. Rev.* **72**, 449 489.

Juul S. E. (2000) Nonerythropoietic roles of erythropoietin in the fetus and neonate. *Clin. Perinatol.* **27**, 527 541.

Juul S. E., Harcum J., Li Y. and Christensen R. D. (1997) Erythropoietin is present in the cerebrospinal fluid of neonates. *J. Pediatr.* **130**, 428 430.

Juul S. E., Anderson D. K., Li Y. and Christensen R. D. (1998) Erythropoietin and erythropoietin receptor in the developing human central nervous system. *Pediatr. Res.* **43**, 40 49.

Juul S. E., Stallings S. A. and Christensen R. D. (1999a) Erythropoietin in the cerebrospinal fluid of neonates who sustained CNS injury. *Pediatr. Res.* **46**, 543 547.

Juul S. E., Yachnis A. T., Rojiani A. M. and Christensen R. D. (1999b) Immunohistochemical localization of erythropoietin and its receptor in the developing human brain. *Pediatr. Dev. Pathol.* **2**, 148 158.

Juul S. E., McPherson R. J., Farrell F. X., Jolliffe L., Ness D. J. and Gleason C. A. (2004) Erythropoietin concentrations in cerebrospinal fluid of nonhuman primates and fetal sheep following high-dose recombinant erythropoietin. *Biol. Neonate* **85**, 138 144.

Kawakami M., Sekiguchi M., Sato K., Kozaki S. and Takahashi M. (2001) Erythropoietin receptor-mediated inhibition of exocytotic glutamate release confers neuroprotection during chemical ischemia. *J. Biol. Chem.* **276**, 39 469 39 475.

Kumral A., Ozer E., Yilmaz O., Akhisaroglu M., Gokmen N., Duman N., Ulukus C., Genc S. and Ozkan H. (2003) Neuroprotective effect of erythropoietin on hypoxic-ischemic brain injury in neonatal rats. *Biol. Neonate* **83**, 224 228.

Leist M. and Jäättelä M. (2001) Four deaths and a funeral: from caspases to alternative mechanisms. *Nat. Rev. Mol. Cell Biol.* **2**, 589 598.

Marti H. H., Wenger R. H., Rivas L. A., Straumann U., Digicaylioglu M., Henn V., Yonekawa Y., Bauer C. and Gassmann M. (1996) Erythropoietin gene expression in human, monkey and murine brain. *Eur. J. Neurosci.* **8**, 666 676.

Marti H. H., Bernaudin M., Petit E. and Bauer C. (2000) Neuroprotection and angiogenesis: dual role of erythropoietin in brain ischemia. *News Physiol. Sci.* **15**, 225 229.

Matsushita H., Johnston M. V., Lange M. S. and Wilson M. A. (2003) Protective effect of erythropoietin in neonatal hypoxic ischemia in mice. *Neuroreport* **14**, 1757 1761.

Merry D. E., Veis D. J., Hickey W. F. and Korsmeyer S. J. (1994) bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS. *Development* **120**, 301 311.

Mori M., Uchida M., Watanabe T., Kirito K., Hatake K., Ozawa K. and Komatsu N. (2003) Activation of extracellular signal-regulated kinases ERK1 and ERK2 induces Bcl-xL up-regulation via inhibition of caspase activities in erythropoietin signaling. *J. Cell Physiol.* **195**, 290 297.

Morikawa Y., Nishida H., Misawa K., Nosaka T., Miyajima A., Senba E. and Kitamura T. (1998) Induction of synaptosomal-associated protein-23 kD (SNAP-23) by various cytokines. *Blood* **92**, 129 135.

Morishita E., Masuda S., Nagao M., Yasuda Y. and Sasaki R. (1997) Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents *in vitro* glutamate-induced neuronal death. *Neuroscience* **76**, 105 116.

Nagy G., Reim K., Matti U., Brose N., Binz T., Rettig J., Neher E. and Sorensen J. B. (2004) Regulation of releasable vesicle pool sizes by protein kinase A-dependent phosphorylation of SNAP-25. *Neuron* **41**, 417 429.

Ni B., Wu X., Su Y., Stephenson D., Smalstig E. B., Clemens J. and Paul S. M. (1998) Transient global forebrain ischemia induces a prolonged expression of the caspase-3 mRNA in rat hippocampal CA1 pyramidal neurons. *J. Cereb. Blood Flow Metab.* **18**, 248 256.

Ota K., Yakovlev A. G., Itaya A., Kameoka M., Tanaka Y. and Yoshihara K. (2002) Alteration of apoptotic protease-activating factor-1 (APAF-1)-dependent apoptotic pathway during development of rat brain and liver. *J. Biochem. (Tokyo)* **131**, 131 135.

Raff M. C., Barres B. A., Burne J. F., Coles H. S., Ishizaki Y. and Jacobson M. D. (1993) Programmed cell death and the control of cell survival: lessons from the nervous system. *Science* **262**, 695 700.

Renzi M. J., Farrell F. X., Bittner A., Galindo J. E., Morton M., Trinh H. and Jolliffe L. K. (2002) Erythropoietin induces changes in gene expression in PC-12 cells. *Brain Res. Mol. Brain Res.* **104**, 86 95.

Rice J. E., Vannucci R. C. and Brierley J. B. (1981) The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Ann. Neurol.* **9**, 131 141.

Ruscher K., Freyer D., Karsch M., Isaev N., Megow D., Sawitzki B., Priller J., Dirnagl U. and Meisel A. (2002) Erythropoietin is a paracrine mediator of ischemic tolerance in the brain: evidence from an *in vitro* model. *J. Neurosci.* **22**, 10 291 10 301.

Sadamoto Y., Igase K., Sakanaka M., Sato K., Otsuka H., Sakaki S., Masuda S. and Sasaki R. (1998) Erythropoietin prevents place navigation disability and cortical infarction in rats with permanent occlusion of the middle cerebral artery. *Biochem. Biophys. Res. Commun.* **253**, 26 32.

Sakanaka M., Wen T. C., Matsuda S., Masuda S., Morishita E., Nagao M. and Sasaki R. (1998) *In vivo* evidence that erythropoietin protects neurons from ischemic damage. *Proc. Natl Acad. Sci. USA* **95**, 4635 4640.

Shan R., Price J. O., Gaarde W. A., Monia B. P., Krantz S. B. and Zhao Z. J. (1999) Distinct roles of JNKs/p38 MAP kinase and ERKs in apoptosis and survival of HCD-57 cells induced by withdrawal or addition of erythropoietin. *Blood* **94**, 4067 4076.

Siren A. L., Fratelli M., Brines M. *et al.* (2001) Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. *Proc. Natl Acad. Sci. USA* **98**, 4044 4049.

Sun Y., Zhou C., Polk P., Nanda A. and Zhang J. H. (2004) Mechanisms of erythropoietin-induced brain protection in neonatal hypoxia-ischemia rat model. *J. Cereb. Blood Flow Metab.* **24**, 259 270.

Tan C. C., Eckardt K. U., Firth J. D. and Ratcliffe P. J. (1992) Feedback modulation of renal and hepatic erythropoietin mRNA in response to graded anemia and hypoxia. *Am. J. Physiol.* **263**, F474 F481.

Vekrellis K., McCarthy M. J., Watson A., Whitfield J., Rubin L. L. and Ham J. (1997) Bax promotes neuronal cell death and is down-regulated during the development of the nervous system. *Development* **124**, 1239 1249.

Volpe J. J. (2001) *Neurology of the Newborn*, 4th edn, p. 995. Saunders, Philadelphia.

Vuong G. L., Weiss S. M., Kammer W., Priemer M., Vingron M., Nordheim A. and Cahill M. A. (2000) Improved sensitivity proteomics by postharvest alkylation and radioactive labelling of proteins. *Electrophoresis* **21**, 2594 2605.

Wang X., Karlsson J. O., Zhu C., Bahr B. A., Hagberg H. and Blomgren K. (2001) Caspase-3 activation after neonatal rat cerebral hypoxia-ischemia. *Biol. Neonate* **79**, 172 179.

Wang X., Zhu C., Qiu L., Hagberg H., Sandberg M. and Blomgren K. (2003) Activation of ERK1/2 after neonatal rat cerebral hypoxia-ischaemia. *J. Neurochem.* **86**, 351 362.

Wen T. C., Sadamoto Y., Tanaka J., Zhu P. X., Nakata K., Ma Y. J., Hata R. and Sakanaka M. (2002) Erythropoietin protects neurons against chemical hypoxia and cerebral ischemic injury by up-regulating Bcl-xL expression. *J. Neurosci. Res.* **67**, 795 803.

Whitaker J. R. and Granum P. E. (1980) An absolute method for protein determination based on difference in absorbance at 235 and 280 nm. *Anal. Biochem.* **109**, 156 159.

Wiessner C., Allegrini P. R., Ekatodramis D., Jewell U. R., Stallmach T. and Gassmann M. (2001) Increased cerebral infarct volumes in polyglobulic mice overexpressing erythropoietin. *J. Cereb. Blood Flow Metab.* **21**, 857 864.

Wolf R. F., Gilmore L. S., Friese P., Downs T., Burstein S. A. and Dale G. L. (1997b) Erythropoietin potentiates thrombus development in a canine arterio-venous shunt model. *Thromb. Haemost.* **77**, 1020 1024.

Wolf R. F., Peng J., Friese P., Gilmore L. S., Burstein S. A. and Dale G. L. (1997a) Erythropoietin administration increases production and reactivity of platelets in dogs. *Thromb. Haemost.* **78**, 1505 1509.

Yager J. Y., Heitjan D. F., Towfighi J. and Vannucci R. C. (1992) Effect of insulin-induced and fasting hypoglycemia on perinatal hypoxic-ischemic brain damage. *Pediatr. Res.* **31**, 138 142.

Yu X., Shacka J. J., Eells J. B. *et al.* (2002) Erythropoietin receptor signalling is required for normal brain development. *Development* **129**, 505 516.

Zhu C., Qiu L., Wang X., Hallin U., Cande C., Kroemer G., Hagberg H. and Blomgren K. (2003) Involvement of apoptosis-inducing factor in neuronal death after hypoxia-ischemia in the neonatal rat brain. *J. Neurochem.* **86**, 306 317.