

# Erythropoietin Is a Paracrine Mediator of Ischemic Tolerance in the Brain: Evidence from an *In Vitro* Model

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In an *in vitro* model of cerebral ischemia (oxygen glucose deprivation, OGD) we investigated whether erythropoietin (EPO) plays a critical role in ischemic preconditioning. We found that EPO time and dose-dependently induced protection against OGD in rat primary cortical neurons. Protection was significant at 5 min and reached a maximum at 48 hr after EPO application. Protection was blocked by the coapplication of a soluble Epo receptor (sEpoR) or an antibody against EpoR (anti-EpoR). Medium transfer from OGD-treated astrocytes to untreated neurons induced protection against OGD in neurons, which was attenuated strongly by the application of sEpoR and anti-EpoR. In contrast, medium transfer from OGD-treated neurons to untreated neurons induced protection against OGD that did not involve EPO. In astrocytes the OGD enhanced the nuclear translocation of hypoxia-inducible factor 1 (HIF-1), the major transcription factor regulating EPO expression. Consequently, transcription of EPO-mRNA was increased in astrocytes after OGD. Cultured neurons express EpoR, and the

Janus kinase-2 (JAK-2) inhibitor AG490 abolished EPO-induced tolerance against OGD. Furthermore, EPO-induced neuroprotection as well as phosphorylation of the proapoptotic Bcl family member Bad was reduced by the phosphoinositide-3 kinase (PI3K) inhibitor LY294002. The results suggest that astrocytes challenged with OGD provide paracrine protective signals to neurons. We provide evidence for the following signaling cascade: HIF-1 is activated rapidly by hypoxia in astrocytes. After HIF-1 activation the astrocytes express and release EPO. EPO activates the neuronal EPO receptor and, subsequently, JAK-2 and thereby PI3K. PI3K deactivates BAD via Akt-mediated phosphorylation and thus may inhibit hypoxia-induced apoptosis in neurons. Our results establish EPO as an important paracrine neuroprotective mediator of ischemic preconditioning.

**Key words:** astrocyte; Bad; hypoxia-inducible factor-1; ischemic preconditioning; Janus kinase-2; neuron; oxygen glucose deprivation; phosphoinositol-3 kinase

In the brain a nonlethal ischemic event can induce tolerance against subsequent, more severe ischemia: “ischemic preconditioning” (IP) or “ischemic tolerance” (Kitagawa et al., 1990). IP is a biphasic phenomenon, with an early short-lasting phase of protection that develops within minutes from the initial ischemic insult and lasts ~2 hr. Subsequently, a cascade of signaling events initiated by the preconditioning stress establishes delayed protection. This delayed phase becomes apparent 12–72 hr after the preconditioning event and lasts for at least 3 d (Perez-Pinzon et al., 1997). Because of its sustained duration and potential clinical relevance (Weih et al., 1999; Moncayo et al., 2000), considerable interest recently has been focused on the delayed phase of IP.

To elucidate the mechanisms of IP, we and others have modeled IP *in vitro*. At 24–78 hr after murine or rat cortical cell cultures were exposed to sublethal oxygen glucose deprivation (OGD), reduced neuronal death to otherwise lethal OGD was observed (Bruer et al., 1997; Grabb and Choi, 1999; Gonzalez-Zulueta et al., 2000). There is evidence that OGD preconditioning is NMDA receptor-dependent (Grabb and Choi, 1999) and

that induction of Ras in an NMDA- and NO-dependent manner is sufficient and necessary for tolerance induction *in vitro* (Gonzalez-Zulueta et al., 2000). Furthermore, the initial signals for triggering preconditioning involve the opening of ATP-sensitive K<sup>+</sup> channels via the activation of adenosine A<sub>1</sub> receptors (Heurteaux et al., 1995; Plamondon et al., 1999). Although most of IP research has focused on intracellular mechanisms, intercellular communication as a route of protective signaling has, in large part, been overlooked. In particular, glial cells, besides participating in the extracellular homeostasis of ions and metabolites in the brain (Ridet et al., 1997), are well known to produce a host of trophic and protective proteins after various stimuli, raising the issue of glial–neuronal protective signaling in IP.

Astrocytes are the main cellular source of the glycoprotein hormone erythropoietin (EPO) in the brain, and low oxygen tension stimulates EPO-mRNA expression in astrocytes. EPO receptor (EpoR) has been detected in neurons as well as in astrocytes (Masuda et al., 1993, 1994; Digicaylioglu et al., 1995; Marti et al., 1996; Liu et al., 1997; Juul et al., 1998; Bernaudin et al., 2000). This may have implications for IP, because EPO has potent neuroprotective properties *in vivo* and *in vitro* (Konishi et al., 1993; Morishita et al., 1997; Sadamoto et al., 1998; Sakanaka et al., 1998; Bernaudin et al., 1999; Brines et al., 2000; Calapai et al., 2000; Sinor and Greenberg, 2000; Sirén et al., 2001). EPO-induced neuroprotection seems to be mediated mainly by anti-apoptotic signaling cascades, which are well established for the

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role of EPO in hematopoiesis (Socolovsky et al., 1999; Lawson et al., 2000). The expression of EPO is regulated by the transcription factor hypoxia-inducible factor-1 (HIF-1) (Semenza, 2000). Recent studies suggest that HIF-1 mediates hypoxia-induced preconditioning in the brain (Ruscher et al., 1998; Zaman et al., 1999; Bergeron et al., 2000), indirectly suggesting a role for EPO in IP.

To address protective astroglial–neuronal signaling and a putative role of EPO herein, we have used purified cell cultures of astrocytes and neurons, because in these systems it is straightforward to separate and identify soluble extracellular factors from intracellular mechanisms by transferring conditioned medium from one cell type or stimulus condition to another. We tested the following hypotheses: (1) hypoxia in astrocytes induces HIF-1, which transactivates EPO synthesis; (2) EPO released into the extracellular space acts as a paracrine endogenous neuroprotectant; and (3) neuroprotection occurs via a cascade of protein phosphorylation that counteracts hypoxia-induced apoptosis.

## MATERIALS AND METHODS

**Cell cultures.** All media and supplements were purchased from Biochrom (Berlin, Germany) unless otherwise noted. Primary neuronal cultures of cerebral cortex were obtained from embryos (E16–E18) of Wistar rats (Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, Germany). Cultures were prepared according to Brewer (1995), with the following modifications: cerebral cortex was dissected, meninges were removed, and tissue was incubated for 15 min in trypsin/EDTA (0.05/0.02% w/v in PBS) at 37°C; the cultures were rinsed twice with PBS and once with dissociation medium (modified Eagle's medium with 10% fetal calf serum, 10 mM HEPES, 44 mM glucose, 100 U penicillin plus streptomycin/ml, 2 mM L-glutamine, 100 IE insulin/l), dissociated by Pasteur pipette in dissociation medium, pelleted by centrifugation ( $210 \times g$  for 2 min at 21°C), redissociated in starter medium [Neurobasal medium with supplemental B27 (Invitrogen, Paisley, UK), 100 U penicillin + streptomycin/ml, 0.5 mM L-glutamine, 25  $\mu$ M glutamate], and plated in 24-well plates or six-well plates in a density of 200,000 cells/cm<sup>2</sup>. Wells were pretreated by incubation with poly-L-lysine (0.5% w/v in PBS) for 1 hr at room temperature and then rinsed with PBS, followed by incubation with coating medium (dissociation medium with 0.03 w/v collagen G) for 1 hr at 37°C; then they were rinsed twice with PBS before the cells were seeded in starter medium. Cultures were kept at 36.5°C and 5% CO<sub>2</sub> and fed beginning from 4 d *in vitro* (DIV) with cultivating medium (starter medium without glutamate) by replacing one-half of the medium twice a week. The cultures were used for experiments after 8 DIV, containing <10% astroglial cells.

Astroglial cell cultures were prepared according to a modified method described by McCarthy and de Vellis (1980). In brief, meninges from cortices of newborn Wistar rats were removed, and the tissue was dissected mechanically and digested in trypsin/EDTA solution (0.05% Trypsin, 0.02% EDTA) at 37°C for 15 min. After digestion the tissue was washed two times in PBS, followed by a mechanical dissociation in DMEM with a pipette. The dissociated cells were seeded in 75 cm<sup>2</sup> flasks (2 brains/flask). Cells were grown in DMEM (10% FCS, 1% penicillin/streptomycin, 2 mM L-glutamine, 0.1% glucose). After 8–10 d the cultures were shaken for 2 hr at 200 rpm to remove microglial cells, and astrocytes were reseeded in subcultures.

**Induction of neuroprotection by OGD-conditioned medium from astrocytes.** Experiments were performed with confluent astrocytes grown in the second subculture. For adaptation of the astrocytes, 72 hr before OGD the astroglial medium (DMEM) was exchanged by neuronal medium (NBM supplemented with B27), as shown in Figure 5A. For astrocyte OGD the culture medium was washed out with PBS, and OGD was induced with a deoxygenated aglycemic solution [DAS; containing (in mM) 143.8 Na<sup>+</sup>, 5.5 K<sup>+</sup>, 1.8 Ca<sup>2+</sup>, 1.8 Mg<sup>2+</sup>, 125.3 Cl<sup>-</sup>, 26.2 HCO<sub>3</sub><sup>-</sup>, 1.0 PO<sub>4</sub><sup>3-</sup>, and 0.8 SO<sub>4</sub><sup>2-</sup>, pH 7.4] in a hypoxic atmosphere (1% oxygen). Hypoxia was generated in a dedicated, humidified gas-tight incubator (Concept 400, Ruskin Technologies, Bridgend, UK) and flushed with gas of the following composition: 5% CO<sub>2</sub>, 85% N<sub>2</sub>, and 10% H<sub>2</sub>. During OGD (180 min) oxygen tensions in the media were below 1 mmHg (polarographic probe, Licox GSM). In control experiments the medium was replaced by basic salt solution [BSS; containing

(in mM) 143.8 Na<sup>+</sup>, 5.5 K<sup>+</sup>, 1.8 Ca<sup>2+</sup>, 1.8 Mg<sup>2+</sup>, 125.3 Cl<sup>-</sup>, 26.2 HCO<sub>3</sub><sup>-</sup>, 1.0 PO<sub>4</sub><sup>3-</sup>, and 0.8 SO<sub>4</sub><sup>2-</sup> plus 4.5 gm/l glucose, pH 7.4] after being washed with PBS, and cells were incubated in a normoxic atmosphere containing 5% CO<sub>2</sub> (see Fig. 5A). Immediately after OGD preconditioning of astrocytes the solutions were replaced by fresh NBM supplemented with B27. Then 24 hr later the medium was removed from astrocytes ( $25 \text{ ml}/2 \times 10^6$  cells); subsequently, neuronal cultures (8 DIV) were fed with the preconditioned medium (400  $\mu$ l) for 48 hr. In parallel experiments, 2.5  $\mu$ g/ml antibody against erythropoietin receptor (anti-EpoR) or 2.5  $\mu$ g/ml soluble EpoR was added to the astrocyte preconditioned medium before being added to neuronal cultures (see Fig. 5A). For neuronal OGD, immediately before starting OGD the medium was removed and washed out with PBS. Medium from untreated cells was stored at 37°C. Thereafter, OGD was induced with DAS medium in a hypoxic atmosphere. Hypoxia was generated as described above. In control experiments the medium was replaced by BSS (after washing with PBS), and the cells were incubated in a normoxic atmosphere containing 5% CO<sub>2</sub>. Immediately after OGD the hypoxic or basic salt solution was replaced by 25% of stored medium and 75% fresh NBM supplemented with B27. After 24 hr lactate dehydrogenase (LDH) activities were measured in the medium supernatant as a marker of cell death (see Fig. 5A).

**Induction of neuroprotection by OGD-conditioned medium from primary cortical neurons.** Experiments were performed with rat primary cortical neurons cultured for 8 DIV. For OGD preconditioning the culture medium was washed out with PBS, and OGD was induced with DAS medium in a hypoxic atmosphere (see Fig. 6A). Hypoxia was generated as described in the astrocyte preconditioning protocol. In control experiments the medium was replaced by BSS (after being washed with PBS), and the cells were incubated in a normoxic atmosphere containing 5% CO<sub>2</sub> (see Fig. 6A). Immediately after OGD preconditioning of neurons the solutions were replaced by fresh NBM supplemented with B27. Then 24 hr later the medium was removed from neurons ( $30 \text{ ml}/18 \times 10^6$  cells); subsequently, fresh neuronal cultures (8 DIV) were fed with the preconditioned medium (400  $\mu$ l) for 48 hr. In parallel experiments, 2.5  $\mu$ g/ml anti-EpoR antibody or 2.5  $\mu$ g/ml soluble erythropoietin receptor (sEpoR) was added to the neuron preconditioned medium before being added to fresh neuronal cultures (see Fig. 6A). For lethal OGD, immediately before OGD was started, the medium was removed and washed out with PBS. Medium from untreated cells was stored at 37°C. Neurons were OGD-treated as described in the astrocyte-preconditioning protocol. After 24 hr LDH activities were measured in the medium supernatant as a marker of cell death (see Fig. 6A).

**Recombinant human EPO stimulation of neurons.** For pretreatment with EPO, cultured neurons (8 DIV) were incubated in the plating medium containing recombinant human EPO (rhEPO) at final concentrations of 1, 10, or 100 U/l, respectively (Sigma-Aldrich, Deisenhofen, Germany), under normoxic, humidified conditions. At different time points (as indicated in the figures) the culture medium was removed and washed out with PBS to nondetectable levels (data not shown) as measured by an erythropoietin chemiluminescent immunoassay. Medium from untreated cells was stored at 37°C. Neurons were OGD-treated as described in the astrocyte preconditioning protocol. Immediately after OGD the hypoxic or basic salt solution was replaced by 25% of stored medium and 75% fresh NBM supplemented with B27. For post-OGD incubation with EPO the cultured neurons (8 DIV) were OGD-treated for 120 min as described in the astrocyte preconditioning protocol. Immediately after OGD, 100 U/l EPO (final concentration) was added to the replaced medium (25% of stored medium and 75% fresh NBM supplemented with B27). After 24 hr LDH activities were measured in the medium supernatant. In parallel experiments, 2.5  $\mu$ g/ml anti-EpoR or 2.5  $\mu$ g/ml sEpoR was coapplied with 100 U/l rhEPO to the neuronal culture medium and washed out with the NBM culture medium immediately before OGD. The kinase inhibitors LY294002 and AG490 were obtained from Promega (Mannheim, Germany) and Calbiochem (Schwalbach, Germany), respectively.

**Erythropoietin chemiluminescent immunoassay.** EPO concentrations were measured by the EPO Immulite assay according to the manufacturer's instructions (DPC Biermann, Bad Nauheim, Germany).

**Lactate dehydrogenase assay.** At 24 hr after OGD or BSS stimulation cell injury was assessed by using phase-contrast microscopy and by measurement of LDH activity. In neuronal cultures the LDH activity in the medium robustly correlated with the number of damaged cells (Koh and Choi, 1987). LDH release was measured as described previously (Bruer et al., 1997).

**Ethidium bromide and acridine orange staining.** The fluorescent DNA-binding dyes ethidium bromide and acridine orange were used to confirm apoptosis, by visualization of condensed and fragmented chromatin, and to distinguish necrotic from apoptotic cells. Whereas acridine orange is membrane-permeable and stains living cells, ethidium homodimer cannot penetrate intact cellular membranes and stains the nucleus of cells with a disrupted membrane. Therefore, stained cells with a regular-sized green fluorescent nucleus represents living cells. Early apoptotic cells have a green fluorescent condensed, shrunken, or fragmented nucleus; late apoptotic cells have a red fluorescent condensed, shrunken, or fragmented nucleus. Necrotic cells exhibit a red fluorescent regular-sized or increased nucleus. After treatment as indicated, primary cortical neurons were incubated in 2  $\mu\text{g}/\text{ml}$  AO (Sigma-Aldrich) and 2  $\mu\text{g}/\text{ml}$  EB (Sigma-Aldrich) for 5 min before imaging and cell counting, using a fluorescence microscope with a standard fluorescein excitation filter (Leica, Heerbrugg, Switzerland).

**Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling assay.** For terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay, the cells were air-dried and fixed with freshly prepared fixation solution (4% PFA in PBS, pH 7.4). TUNEL was performed by using a fluorescence *in situ* Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

**DNA laddering.** DNA was extracted by using a modified commercial Easy-DNA Kit (Invitrogen BV, Breda, Netherlands). DNA (2  $\mu\text{g}$ ) was separated on a 2% agarose gel and stained by ethidium bromide. DNA fragments were analyzed with a fluorescence imager (Typhoon, Amersham Biosciences, Freiburg, Germany).

**Immunocytochemistry.** For immunocytochemical analysis the neurons were plated on glass coverslips and fixed with 4% paraformaldehyde for 10 min. After pretreatment in PBS supplemented with 0.3% Triton X-100 and 10% normal goat serum, the coverslips were incubated overnight with a polyclonal antibody against EpoR (Santa Cruz Biotechnology, Freiburg, Germany) at a dilution of 1:100. After several washes a secondary biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) was applied at a dilution of 1:200 for 30 min. Visualization was achieved via the Vectorstain ABC Elite kit (Vector Laboratories) reacted with 3,3'-diaminobenzidine/ $\text{H}_2\text{O}_2$  (Sigma-Aldrich). Omission of the primary antibody served as a negative control.

**Fluorescent electrophoretic mobility shift assay.** Nuclear extracts were prepared as described previously (Ruscher et al., 2000). For gel shift assays 1 pmol of Cy5-labeled specific probe was incubated with 25  $\mu\text{g}$  of protein of nuclear extracts in binding buffer BBN [containing (in mM) 20 HEPES, 50 KCl, 1 EDTA, and 1 DTT plus 25 ng poly(dI)-poly(dC), 10% glycerol] for 15 min at room temperature. The following Cy5-labeled specific double-stranded probes were used for HIF-1 and signal transducer and activator of transcription (STAT1), STAT3, and STAT5 gel shift assays: HIF-1, 5'-Cy5-AGTTGAGGGGACTTCCAGGC-3'; STAT1, 5'-Cy5-CATGTTATGCATATTCCTGTAAGTG-3'; STAT3, 5'-Cy5-GATCCTTCTGGGAATTCCTAGATC-3', and STAT5 5'-Cy5-AGATTTCTAGGAATTCATCC-3'. Specificity was confirmed by the addition of a 50-fold excess of either unlabeled specific competitor (specific probes without the Cy5 label) or unlabeled nonspecific competitor (5'-GACGTATGAGTCAGTCCA-3'). HIF-1 supershifts were performed by using a polyclonal antibody against HIF-1 $\alpha$  (Santa Cruz Biotechnology). Ten microliters of the mixture were separated on a 5% nondenaturing polyacrylamide gel at 4°C in 1 $\times$  TBE (90 mM Tris-borate and 2 mM EDTA, pH 8.3), using an external temperature-regulated ALF-Express DNA sequencer. Gels were analyzed directly by ALFwin and Allelix software (Amersham Biosciences) as described previously (Ruscher et al., 2000).

**Quantitative competitive RT-PCR of EPO mRNA.** Total cellular RNA was isolated from  $\sim 10^6$  cells according to a modified method described by Chomczynski and Sacchi (1987), using Trizol reagent (Life Technology, Karlsruhe, Germany). Contaminating DNA was removed by incubating the RNA with DNase (Promega, Madison, WI) as recommended by the supplier. Integrity of the RNA was verified by gel electrophoresis, and RNA quantity was determined by optical density measurement. Reverse transcription of extracted RNA was performed by standard procedures, using Maloney murine leukemia virus reverse transcriptase (Promega). Using the housekeeping gene  $\beta$ -actin as an internal standard for RNA preparation and reverse transcriptase reaction, we performed quantitative competitive RT-PCR as described recently (Prass et al., 2002).

**Quantitative real time RT-PCR of Bag-1, Bcl-X<sub>L</sub>, Bcl-2, Bax, and Bad.** For evaluation of Bag-1, Bcl-X<sub>L</sub>, Bcl-2, Bax, and Bad gene expression, real time TaqMan RT-PCR was performed. At first, total RNA was prepared from cultured cells and was reverse transcribed into cDNA as described above for EPO RT-PCR. Expression of each sample was normalized on the basis of its  $\beta$ -actin mRNA content. In TaqMan assays a specific oligonucleotide probe is annealed to a target sequence located between the two primer binding sites. The probe is labeled with a reporter dye (FAM) at the 5' end and a quencher dye at the 3' end (TAMRA). The real-time PCR amplifications were performed in 25  $\mu\text{l}$  reaction volumes containing 1  $\mu\text{l}$  cDNA, 2.5  $\mu\text{l}$  10 $\times$  PCR buffer, 200  $\mu\text{M}$  each dATP, dCTP, dGTP, 400  $\mu\text{M}$  dUTP, 5 mM MgCl<sub>2</sub>, and 0.25 U Ampli-Taq DNA polymerase and AmpErase uracil *N*-glycosylase (UNG). The thermal cycling conditions included 2 min at 50°C and 10 min at 95°C. Thermal cycling proceeded with 40 cycles of 95°C for 0.5 min and 60°C for 2 min. All reactions were performed in duplicate; for amplification and detection an ABI Prism 7700 Sequence Detection System (PerkinElmer Applied Biosystems, Emeryville, CA) was used. Real-time data were analyzed with Sequence Detection Systems software, version 1.7. Each run contains both negative (no template) and positive controls. The following sequence-specific primers were used in RT-PCR. For  $\beta$ -actin: forward, 5'-GTACAACCTCCTTGCAGCTCCT-3'; reverse, 5'-TTGTGCGACGACGAGCGC-3'; probe, 5'-CGCCACCAGTTCGCCATGGAT-3'. For Bag-1: forward, 5'-CAGC-TAACCACCTGGAAGAGTTG-3'; reverse, 5'-GAGCTCCGCTTGT-AATTCCTT-3'; probe, 5'-TTCTGACATCCAGCAGGGTCTTCTGGC-3'. For Bcl-X<sub>L</sub>: forward, 5'-GGTGAGTCGGATTGCAAGTTG-3'; reverse, 5'-GTAGAGATCCACAAAAGTGTCCAG-3'; probe, 5'-CCTGATGACACCTAGAGCCTTGGATCC-3'. For Bcl-2: forward, 5'-TGAACCGGCATCTGCACA-3'; reverse, 5'-CAGAGGTCGCAT-GCTGGG-3'; probe, 5'-AACGGAGGCTGGGATGCCTTTGTG-3'. For Bax: forward, 5'-GCGTGGTTGCCCTCT TCACTT-3'; reverse, 5'-AGCAGCCGTCACGGAG-3'; probe, 5'-CAAAGTGGTGCTCAAG-GCCCTGTG-3'. For Bad: forward, 5'-CAGGCAGCCAACAA-CAGTCA-3'; reverse, 5'-CGTGGGTACGAAGTGTGG-3'; probe, 5'-CATGGAGGCGCTGGGACTATGGAGA-3'.

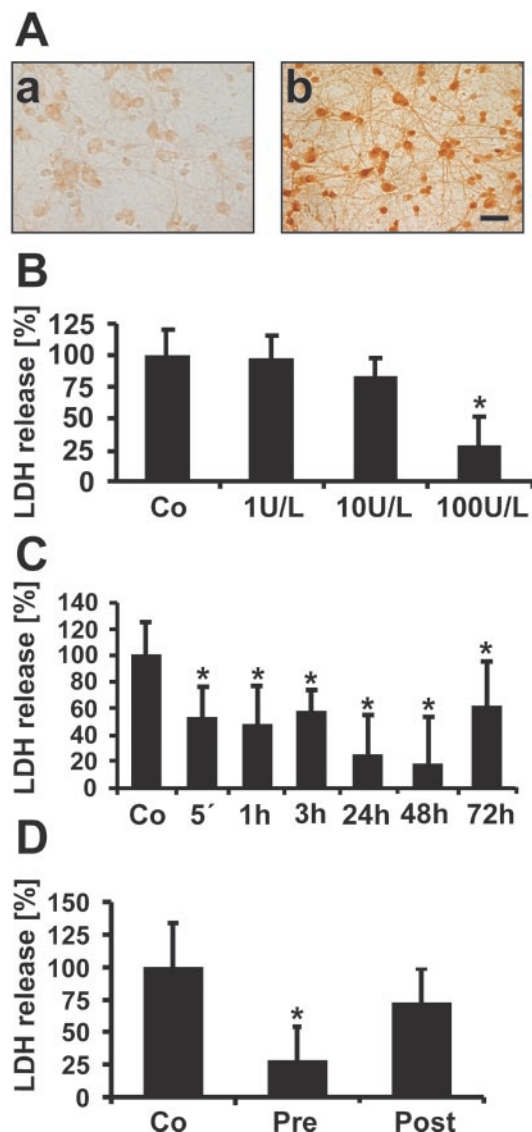
**Western blotting.** Cells were harvested in cell lysis buffer [containing (in mM) 20 Tris, pH 7.5, 150 NaCl, 1 EDTA, 1 EGTA, 2.5 sodium pyrophosphate, 1  $\beta$ -glycerol phosphate, 1 Na<sub>3</sub>VO<sub>4</sub>, and 1 PMSF plus 1% Triton X-100, 1  $\mu\text{g}/\text{ml}$  leupeptin]. After incubation on ice for 15 min and centrifugation at 18,000  $\times$  g at 4°C for 10 min, whole protein concentrations were determined by the BCA assay (Pierce, Rockford, IL), using bovine serum albumin as a standard. Cell lysates were diluted in SDS sample buffer (final concentrations: 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 0.1% bromophenol blue), and the mixture was boiled for 5 min. Protein (30  $\mu\text{g}$ ) was separated on a 10% SDS-polyacrylamide gel. Blocking was performed onto polyvinylidene difluoride membranes with blocking buffer (20 mM Tris, 136 mM NaCl, pH 7.6, 0.1% Tween 20, 5% nonfat dry milk), and detected by using primary polyclonal antibodies against Bad, phospho-Bad (Ser<sup>136</sup>), phospho-p38 mitogen-activated protein kinase (MAPK), and phospho-p44/42 MAPK (diluted 1:1000; all purchased from New England Biolabs, Frankfurt, Germany) and a polyclonal antibody against EPO (diluted 1:1000; Santa Cruz Biotechnology). After incubation overnight at 4°C the signals were obtained by binding of a secondary anti-rabbit HRP-linked antibody and were visualized on x-ray films (Hyperfilm, Amersham Biosciences), using a chemiluminescence kit (New England Biolabs). These experiments were repeated three times with similar results.

**Akt kinase assay.** Cortical neurons were treated with 100 U/l rhEPO. In parallel experiments, 2.5  $\mu\text{g}/\text{ml}$  sEpoR or 10  $\mu\text{M}$  LY294002 was coapplied with 100 U/l rhEPO to the neuronal culture medium. After incubation for 30 min the cells were harvested in lysis buffer, and an Akt kinase assay was performed according to the instruction manual of the supplier (New England Biolabs).

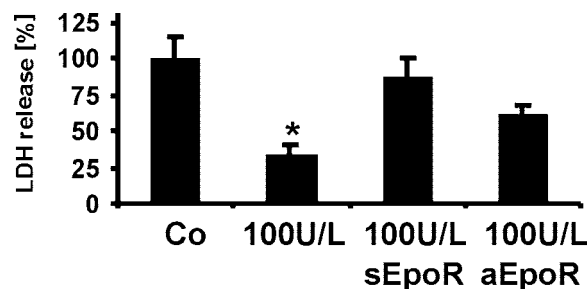
## RESULTS

### rhEPO time- and dose-dependently protects neurons from OGD

Cultured rat cortical neurons expressed EpoR (Fig. 1A). rhEPO induced tolerance against OGD in a dose-dependent manner. Primary cortical neurons were pretreated with different concentrations of rhEPO (1, 10, 100 U/l) for 48 hr. As illustrated in Figure 1B, only the highest concentration of rhEPO afforded



**Figure 1.** Primary cortical neurons express the erythropoietin receptor (EpoR), and human recombinant EPO (rhEPO) dose- and time-dependently induces tolerance against OGD in primary cortical neurons. *A*, Primary cortical neurons express EpoR; expression of EpoR was analyzed by immunocytochemistry in primary cultures of cortical neurons. *a*, Unstained control (omission of primary antibody). *b*, Expression of EpoR on neurons after staining with a polyclonal antibody against EpoR (magnification, 400 $\times$ ). Scale bar, 50  $\mu$ m. *B*, rhEPO dose-dependently induces tolerance against OGD in primary cortical neurons. Before 120 min of OGD primary cortical neurons were pretreated for 48 hr with 1, 10, or 100 U/l rhEPO, respectively. rhEPO (100 U/l) significantly protected primary cortical neurons from cell death. *C*, rhEPO time-dependently induces tolerance against OGD in primary cortical neurons. Pretreatment with 100 U/l rhEPO for the indicated intervals induced a fast and prolonged neuroprotection. After 5 min of EPO exposure the neurons already were protected from OGD-induced cell death by 50%. The maximum of protection was observed after a pretreatment period of 24 and 48 hr. *D*, Compared with EPO-untreated OGD control neurons (*Co*), preincubation of neurons with 100 U/l EPO for 48 hr resulted in a statistically significant reduction of cell death 24 hr after lethal OGD (*Pre*), whereas post-treatment with 100 U/l EPO immediately after lethal OGD was not neuroprotective (*Post*). Cell death in *B–D* was measured as LDH release into the medium for 24 hr after OGD. Data were obtained from three independent experiments with 16 cell cultures each; data were normalized and presented as means  $\pm$  SD. Multiple comparisons (Dunn's method) were performed after Kruskal–Wallis one-way ANOVA on ranks ( $*p > 0.05$  vs *Co*). Normoxic (BSS) stimulation was set to 0% cell death and OGD stimulation (*Co*) to 100% cell death, respectively.



**Figure 2.** EPO-induced neuroprotection is mediated by interaction with the cognate receptor of EPO. Neuroprotection induced by pretreatment with 100 U/l rhEPO 48 hr before lethal OGD (120 min) was inhibited by the coapplication of either a soluble erythropoietin receptor (*sEpoR*) or an antibody against the erythropoietin receptor (anti-EpoR, *aEpoR*). Cell death was measured as LDH release into the medium for 24 hr after OGD. Data were obtained from three independent experiments with 16 cell cultures each; data were normalized and presented as means  $\pm$  SD. Multiple comparisons (Dunn's method) were performed after Kruskal–Wallis one-way ANOVA on ranks ( $*p > 0.05$  vs all other groups). Normoxic (BSS) stimulation was set to 0% cell death and OGD stimulation (*Co*) to 100% cell death, respectively.

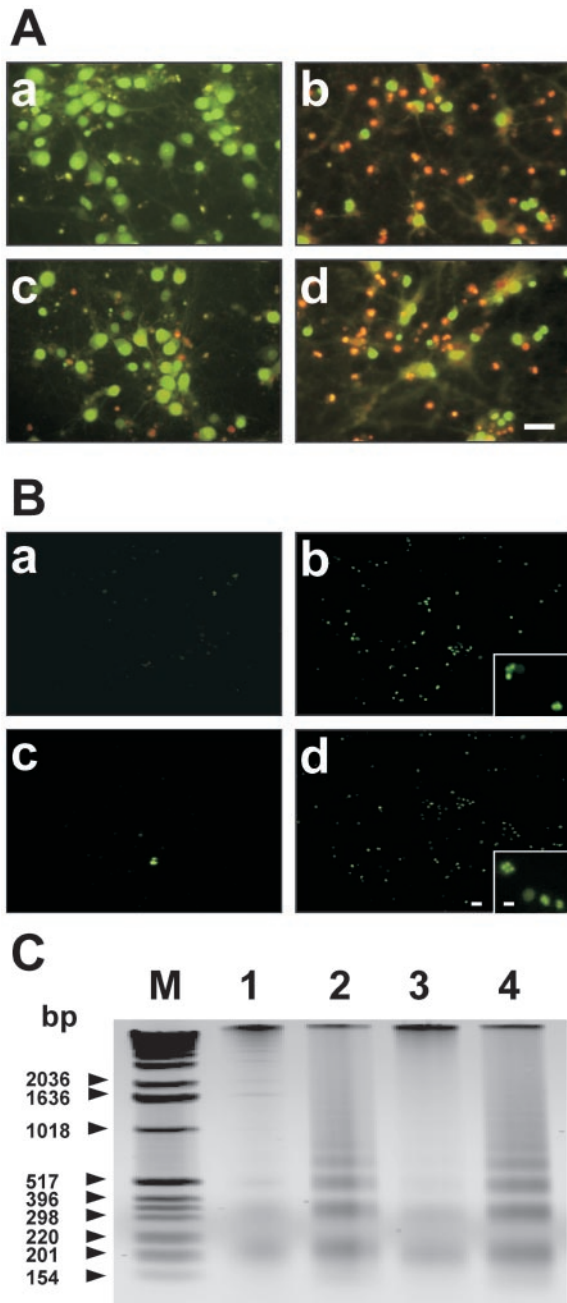
statistically significant neuronal survival after 120 min of OGD, as assessed 24 hr later by LDH assay (75% protection).

To examine the time dependence of neuroprotection by EPO, we pretreated neurons with 100 U/l rhEPO from 5 min to 72 hr before OGD. Surprisingly, even a 5 min incubation period already afforded tolerance against OGD in neurons (Fig. 1*C*). The highest levels of protection were achieved after 48 hr of incubation with rhEPO. Longer intervals were associated with a decreasing level of protection, most probably related to a reduced viability of the cultures because of the prolonged cultivation period. In contrast to EPO preincubation, the addition of EPO to primary cortical neurons immediately after lethal OGD (120 min) had no statistically significant neuroprotective effect (Fig. 1*D*).

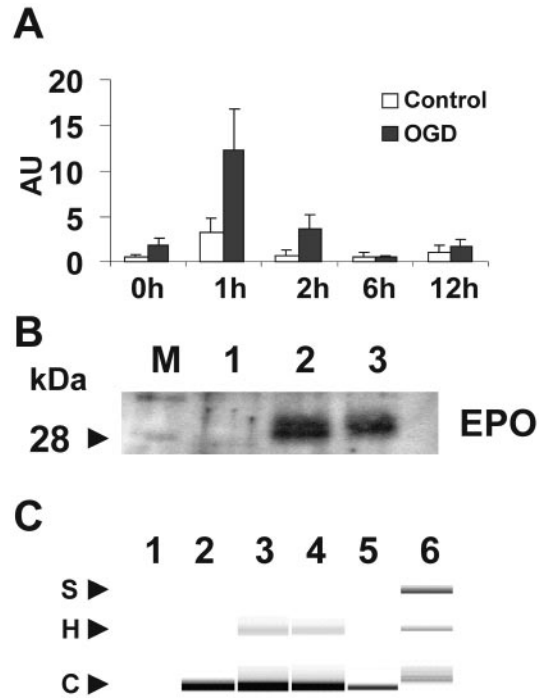
To test whether neuroprotection is mediated by the specific interaction of EPO with its cognate receptor, we coapplied 100 U/l rhEPO either with a soluble EPO receptor (*sEpoR*) or with an antibody against the EPO receptor (anti-EpoR). Both *sEpoR* or anti-EpoR blocked EPO-induced ischemic tolerance (Fig. 2). EPO-induced protection and specific blockade of this protection are illustrated by representative fluorescent micrographs of OGD-challenged neurons stained with ethidium bromide/acridine orange (Fig. 3*A*). In accordance with our previous results (Ruscher et al., 1998), the predominant cell death in our OGD model was apoptosis. Using a TUNEL assay, we demonstrated that rhEPO blocks OGD-induced neuronal apoptosis (Fig. 3*B*). To substantiate the anti-apoptotic effect of EPO further, we analyzed its effect on DNA fragmentation. Preincubation with 100 U/l EPO significantly reduced DNA fragmentation induced by OGD (Fig. 3*C*). *sEpoR* as well as anti-EpoR was able to block the anti-apoptotic effect of EPO (Fig. 3*B,C*).

### Erythropoietin produced by astrocytes mediates neuroprotection against oxygen glucose deprivation

Astrocytes are the major source of EPO production in the brain (see introductory remarks). We therefore investigated EPO expression in astrocytes exposed to OGD for 180 min. Pilot experiments indicated that the degree of EPO induction in astrocytes after OGD was not higher at 180 min compared with 60 min. At 48 hr after OGD the astrocytes showed no reduced viability when analyzed for morphological changes or lactate dehydrogenase



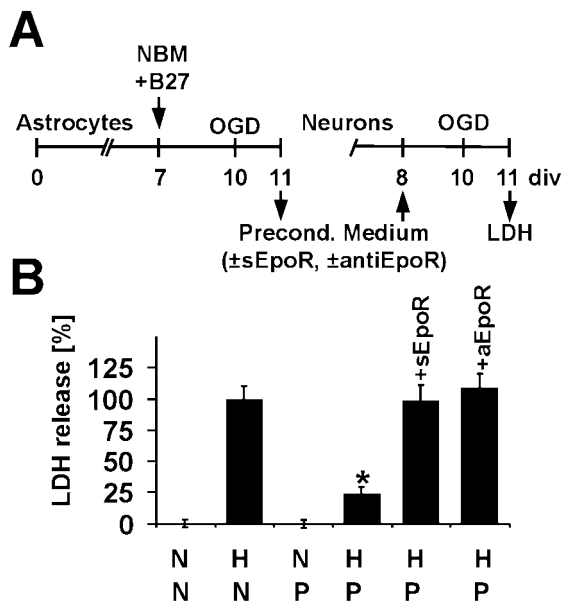
**Figure 3.** Fluorescence microscopy (*A*), TUNEL assay (*B*), and DNA laddering (*C*) demonstrate the neuroprotective effect of EPO in OGD-induced neuronal apoptosis. Shown are micrographs of ethidium bromide/acidine orange-stained (*A*; magnification 400 $\times$ ) and TUNEL-stained (*B*; magnification 200 $\times$ ) primary cortical neurons 24 hr after OGD with or without EPO pretreatment. *a*, Normoxic control; *b*, OGD for 120 min without EPO pretreatment; *c*, OGD for 120 min with EPO pretreatment. Cultured neurons were stimulated by 100 U/l rhEPO 48 hr before 120 min of OGD. *d*, OGD for 120 min with EPO pretreatment and block by anti-EpoR. Stimulation of cultured neurons by 100 U/l rhEPO 48 hr before 120 min of OGD was blocked by the coapplication of 2.5  $\mu$ g/ml anti-EpoR. The insets (magnification 400 $\times$ ) in micrographs *Bb* and *Bd* demonstrate apoptotic bodies. Scale bars: (in *d*) *a*–*d*, 50  $\mu$ m; inset, 20  $\mu$ m. *C*, OGD-induced DNA fragmentation was abolished by pretreatment with EPO. DNA fragmentation was analyzed 24 hr after lethal OGD (120 min) of primary cortical neurons with or without EPO pretreatment. Lane *M*, 1 kb ladder; lane 1, BSS-treated cells; lane 2, OGD-treated cells; lane 3, OGD-treated cells preincubated with 100 U/l rhEPO 48 hr before OGD; lane 4, OGD-treated cells preincubated with 100 U/l rhEPO and 2.5  $\mu$ g/ml sEpoR 48 hr before OGD.



**Figure 4.** Astrocytic HIF-1 activation and erythropoietin expression are sequential events that follow oxygen glucose deprivation. *A*, Astrocytes produce erythropoietin in response to OGD. Astrocytes were stimulated by OGD or control conditions for 180 min. Shown are different time points after OGD cells were harvested and total RNA was isolated and reverse transcribed. Using a quantitative competitive RT-PCR approach and the housekeeping gene  $\beta$ -actin as an internal standard, we determined EPO mRNA expression. Data were obtained from three independent experiments, presented as the means  $\pm$  SD of arbitrary units (AU). *B*, In accordance with the mRNA expression of EPO, the protein was detectable 60 min after OGD. Shown are Western blot analysis from control astroglial cultures (lane 1), OGD-stimulated cultures (lane 2), and positive control (lane 3). The molecular size of the protein standard (lane *M*) is indicated. *C*, Induction of astroglial EPO expression is associated with the preceding induction of HIF-1 binding activity. Astrocytes were stimulated by OGD or control conditions for 180 min. Nuclear extracts were prepared immediately, and 30  $\mu$ g of each was tested for HIF-1 DNA binding activities, using an EMSA approach. Shown are probes without nuclear extract (lane 1), probes and nuclear extracts from BSS-stimulated astrocytes (lane 2), probes and nuclear extracts from OGD-stimulated astrocytes (lane 3), probes and nuclear extract from OGD-stimulated astrocytes and the addition of an unspecific competitor (lane 4; 50-fold), probes and nuclear extract from OGD-stimulated astrocytes and the addition of a specific competitor (lane 5; 50-fold), and probes and nuclear extract from OGD-stimulated astrocytes and the addition of a specific antibody against HIF-1 $\alpha$  (lane 6). *C*, Constitutive DNA binding activity; *H*, specific HIF-1 DNA complex; *S*, supershifted HIF-1 DNA complex.

release (data not shown). For measurement of EPO mRNA expression we used a competitive RT-PCR approach. In response to OGD the astrocytes very rapidly express EPO mRNA. Already at 60 min after OGD the expression reached a maximum with a 12-fold induction over baseline (Fig. 4*A*). This was accompanied by a strong induction of EPO protein (Fig. 4*B*). Control was defined as astrocytes treated with basic salt solution under normoxic conditions, whereas baseline expression was taken from astrocytes before all manipulation (see Materials and Methods).

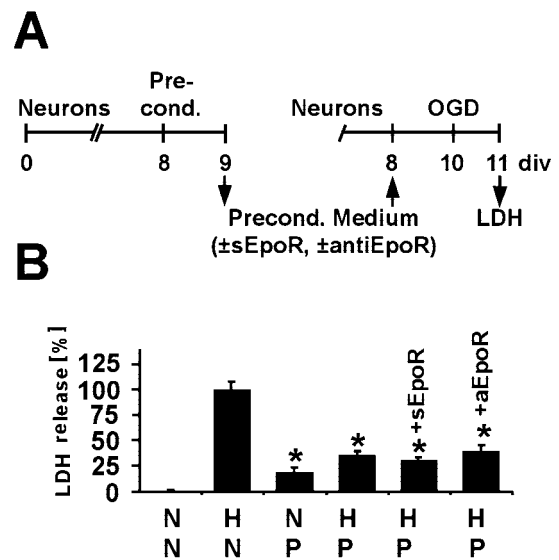
Because EPO expression is controlled mainly by the transcription factor HIF-1 (Semenza, 2000), we investigated whether the activation of HIF-1 precedes EPO expression. Using a fluorescent-based electrophoretic mobility shift assay (fEMSA; Ruscher et al., 2000), we observed marked HIF-1 DNA binding



**Figure 5.** Preconditioned medium from astrocytes induces ischemic tolerance in cortical neurons. *A*, Experimental paradigm. Astrocytes were stimulated for 180 min either by OGD (preconditioning) or with BSS medium under normoxia (no preconditioning), and media were harvested 24 hr later. *B*, Neuronal cultures, pretreated with medium either from OGD-stimulated (preconditioning, *P*, bottom row) or from BSS-stimulated (no preconditioning, *N*, bottom row) astrocytes for 48 hr, were exposed to OGD (*H*, top row) or BSS (*N*, top row) for 120 min, respectively. Cell death, measured as LDH release into the medium for 24 hr after OGD, was reduced significantly by OGD-conditioned medium from astrocytes. This neuroprotection was diminished by 2.5  $\mu$ g/ml sEpoR and 2.5  $\mu$ g/ml anti-EpoR, indicating that EPO is the neuroprotective factor. Data were obtained from three independent experiments with 16 cell cultures each; data were normalized and presented as means  $\pm$  SD. Multiple comparisons (Dunn's method) were performed after Kruskal–Wallis one-way ANOVA on ranks ( $*p > 0.05$  vs *N/H*, *P/H* + sEpoR, or *P/H* + aEpoR). *N* represents normoxic (BSS) and *H* represents hypoxic (OGD) treatment. *N/N* treatment was set to 0% cell death and *N/H* treatment to 100% cell death, respectively.

activity in nuclear extracts from astrocytes after OGD stimulation for 180 min (Fig. 4*C*). The specificity of the HIF-1 binding was tested by competition experiments that used an unlabeled HIF-1 probe or nonspecific probe and was tested by supershift experiments, using an antibody against HIF-1 $\alpha$  (Fig. 4*C*).

We next investigated whether OGD-treated astrocytes release a factor capable of protecting neurons from OGD and whether this factor is EPO. Astrocytes were subjected to 180 min of OGD, after which they were allowed to condition medium for 24 hr. Subsequently, conditioned medium was transferred to neuron-enriched cultures, and the neurons were exposed to this medium for 48 hr. Conditioned medium was washed out before the neurons were exposed to OGD of 120 min, which is lethal for ~75% of neurons. Preincubation with the OGD-conditioned astrocyte medium protected neurons from OGD (Fig. 5*A,B*). LDH assay 24 hr after OGD demonstrated a 75% increase in neuronal viability compared with “control-conditioned” medium. Medium transfer-induced neuronal protection was blocked completely by sEpoR or anti-EpoR, indicating that EPO is the neuroprotective factor released by OGD-stimulated astrocytes (Fig. 5*A,B*). This was verified by using an EPO immunoassay. The OGD-conditioned astrocyte medium contained 258 U/l EPO, which is comparable with the neuroprotective concentrations of rhEPO in this and

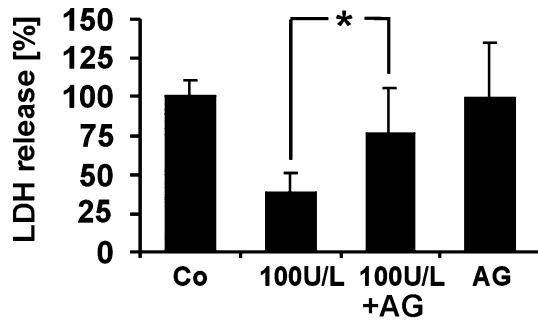


**Figure 6.** Neuro-neuronal preconditioning is not mediated by erythropoietin. *A*, Experimental paradigm. Cortical neurons were stimulated either by OGD for 60 min (preconditioning) or with BSS medium under normoxia (no preconditioning), and both media were harvested 24 hr later. *B*, Neuronal cultures, pretreated with medium either from OGD-stimulated (preconditioning, *P*, bottom row) or from BSS-stimulated (no preconditioning, *N*, bottom row) cortical neurons for 48 hr, were exposed to OGD (*H*, top row) or BSS (*N*, top row) for 120 min. Cell death, measured as LDH release into the medium for 24 hr after OGD, was reduced significantly in neuronal cultures treated with OGD-conditioned medium from neurons. However, this neuroprotection was diminished neither by 2.5  $\mu$ g/ml sEpoR nor by 2.5  $\mu$ g/ml anti-EpoR, excluding EPO as the neuroprotective factor. Data were obtained from three independent experiments with 16 cell cultures each; data were normalized and presented as means  $\pm$  SD. Multiple comparisons (Dunn's method) were performed after Kruskal–Wallis one-way ANOVA on ranks ( $*p > 0.05$  vs *N/H* treatment). *N* represents normoxic (BSS) and *H* represents hypoxic (OGD) treatment. *N/N* treatment was set to 0% cell death and *N/H* treatment to 100% cell death, respectively.

other studies. sEpoR and anti-EpoR had no effect of their own on neuronal cell viability (data not shown).

### Neuron-to-neuron transfer of protection is not mediated by EPO

We have shown previously that primary neurons in culture can be preconditioned by OGD in the presence of <10% astrocytes (Bruer et al., 1997; Ruscher et al., 1998). We therefore investigated whether EPO also is involved in OGD preconditioning of enriched neurons in culture. Neurons were subjected to a preconditioning interval of OGD (90 min) after which they were allowed to condition medium for 24 hr (Fig. 6*A*). Transfer of this medium to naive neuron-enriched cultures and subsequent exposure for 48 hr conferred protection to neurons against a normally lethal OGD (120 min). Thus neurons, like astrocytes, release protective factor(s). However, in this case EPO is not involved, because neither sEpoR nor anti-EpoR affected the protective effect (Fig. 6*A,B*). In line with this finding and in contrast to astrocytes, we observed no induction of EPO-mRNA in neurons after a preconditioning OGD period of 60 min when we used our competitive RT-PCR approach (data not shown). In addition, using an EPO immunoassay, we could not detect any EPO protein in the medium preconditioned by neurons.



**Figure 7.** Erythropoietin-induced neuroprotection is mediated by Janus kinase-2 (JAK-2). Cortical neurons, pretreated with 100 U/l rhEPO and/or AG490 (5  $\mu$ M) for 48 hr, were exposed to 120 min of OGD or BSS. Coapplication of the JAK-2 inhibitor AG490 abolished the neuroprotective effect of 100 U/l rhEPO (100 U/l vs 100 U/l + AG). Neuronal viability under hypoxic (AG) or normoxic (data not shown) conditions is not altered by JAK-2 inhibition alone. Cell death was measured as LDH release into the medium for 24 hr after OGD. Data were obtained from three independent experiments with eight cell cultures each; data were normalized and presented as means  $\pm$  SD ( $*p > 0.05$  vs 100 U/l EPO; Mann–Whitney rank sum test). Normoxic (BSS) stimulation was set to 0% cell death and OGD stimulation (Co) to 100% cell death, respectively.

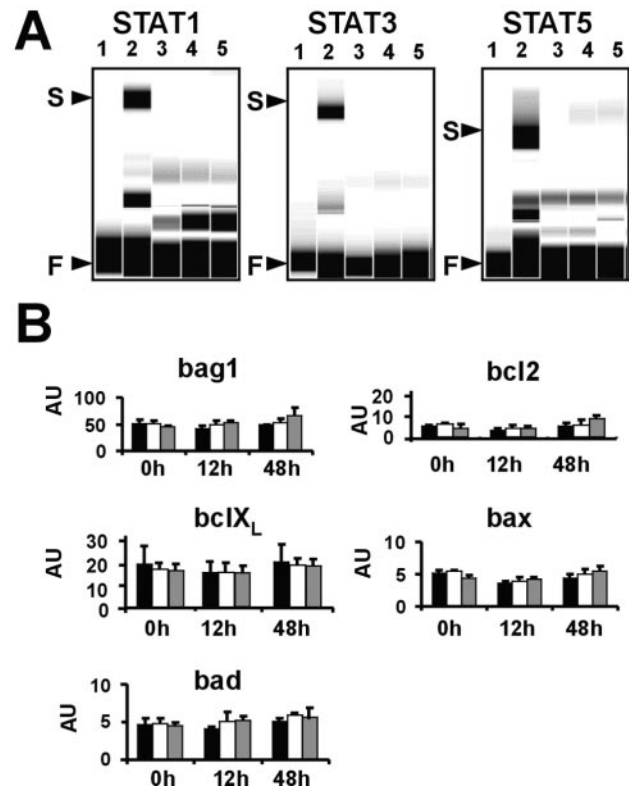
#### Activation of Janus kinase-2 pathway mediates the neuroprotective effect of EPO

Janus kinase-2 (JAK-2) is the key kinase in the signal transduction pathways activated by the EpoR. Therefore, we explored the role of JAK-2-dependent pathways for the anti-apoptotic effect of EPO on OGD-induced apoptosis in neurons. As shown in Figure 7, rhEPO-induced neuroprotection is attenuated significantly in the presence of the specific JAK-2 inhibitor AG490 (5  $\mu$ M). AG490 alone had no effect on neuronal viability. These results strongly suggest a central role of JAK-2 in EPO-mediated neuroprotection.

#### STATs and MAPK pathways are not involved in EPO-mediated neuroprotection

Downstream from JAK-2 at least three different signaling pathways have been implicated in EPO-induced protection from apoptosis: STAT, Ras/MAP kinase, and phosphoinositide-3 kinase (PI3K). First we analyzed the JAK-2/STAT pathway. In non-neuronal tissues EPO can activate the STAT family members STAT1, STAT3, and STAT5 rapidly. Although STAT1, STAT3, and STAT5 have been implicated in the regulation of apoptosis of non-neuronal tissues (Grad et al., 2000), only STAT5 so far has been demonstrated in the anti-apoptotic signaling of Epo (Socolovsky et al., 1999; Constantinescu et al., 2001), whereas only STAT3 is expressed constitutively throughout the brain in glial cells as well as neurons (Murata et al., 2000). Surprisingly, 30 min after rhEPO stimulation we did not find any DNA binding activity of STAT1, STAT3, or STAT5 (Fig. 8A). To exclude that STAT transactivation was short and transient and thus may have been missed, we investigated the mRNA expression of STAT-transactivated anti-apoptotic genes, known to be involved in receptor tyrosine kinase-induced anti-apoptotic pathways. For mRNA quantification we used a real-time RT-PCR approach and the housekeeping gene  $\beta$ -actin as an internal standard. In accordance with our negative STAT data, EPO did not induce Bcl-2, Bcl-X<sub>L</sub>, or Bag-1 transcription in cortical neurons, neither after 12 nor after 48 hr of Epo application (Fig. 8B).

Next we investigated a possible involvement of MAPK pathways, the extracellular-regulated protein kinase (ERK or p42/44),

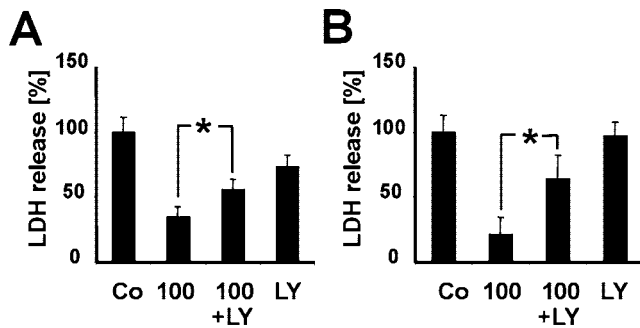


**Figure 8.** JAK-2/STAT pathway is not involved in EPO-mediated neuroprotection. *A*, EPO stimulation of neurons does not activate the transcription factors STAT1, STAT3, and STAT5, as assessed by EMSA. Shown are probes without nuclear extract (lane 1); probes and positive controls for STAT1 from phorbol ester-treated HeLa cells and for STAT3 and STAT5 from phorbol ester-treated K562 cells (lane 2); probes, positive controls, and a 50-fold excess of specific competitor against STAT1, STAT3, or STAT5, respectively (lane 3); probes and extract from untreated neurons (lane 4); and probes and extract from neurons treated by 100 U/l rhEPO for 30 min (lane 5). *F*, Free probe; *S*, specific protein DNA complex. *B*, Transcriptional pattern of anti- and pro-apoptotic Bcl-2 family genes is not altered in neurons by erythropoietin stimulation. Cortical neurons were stimulated by 100 U/l rhEPO for 0, 12, or 48 hr, respectively. EPO-stimulated neurons (black columns) were compared with untreated control neurons (white columns) and with neurons treated with the combination of 100 U/l rhEPO and 2.5  $\mu$ g/ml EpoR (gray columns). Using a quantitative real-time RT-PCR approach and the housekeeping gene  $\beta$ -actin as an internal standard, we found no induction or repression of mRNA expression of Bcl-2, Bcl-X<sub>L</sub>, Bag-1, Bax, and Bad. Data were obtained from three independent experiments, presented as means  $\pm$  SD of arbitrary units (AU).

and p38 mitogen-activated kinase (p38 MAPK) (Herlaar and Brown, 1999). None of these was activated (i.e., phosphorylated) after EPO stimulation (see Fig. 10).

#### EPO-induced neuronal preconditioning is mediated by the activation of a PI3K pathway and subsequent phosphorylation of Bad

We next evaluated whether EPO-induced neuroprotection involves the activation of PI3K. Cortical neurons were treated with 100 U/l rhEPO in the presence of LY294002 (10  $\mu$ M), a cell-permeable specific inhibitor of PI3 kinase. Thereafter, cultured neurons were exposed to a lethal OGD interval either 1 or 24 hr after EPO/LY294002 pretreatment. As shown in Figure 9, the inhibition of PI3K significantly diminished EpoR-mediated neuroprotection in the short (1 hr) as well as in the long (24 hr) time window. In addition, we analyzed the effects on neuronal survival



**Figure 9.** Erythropoietin-induced neuroprotection is mediated by phosphoinositol-3 kinase (PI3K). Pretreatment of neurons for a short (1 hr, *A*) and a prolonged (24 hr, *B*) incubation period with the specific PI3K inhibitor LY294002 (10  $\mu$ M) immediately before the application of 100 U/l EPO partially abolished the neuroprotective effect of EPO against 120 min of OGD (100 U/l vs 100 U/l + LY). Pretreatment with LY294002 alone did not influence neuronal viability under hypoxic (LY) or normoxic (data not shown) conditions. Cell death was measured as LDH release into the medium for 24 hr after OGD. Data were obtained from three independent experiments with eight cell cultures each; data were normalized and presented as means  $\pm$  SD ( $*p > 0.05$  vs 100 U/l EPO; Mann–Whitney rank sum test). Normoxic (BSS) stimulation was set to 0% cell death and OGD stimulation (Co) to 100% cell death, respectively.

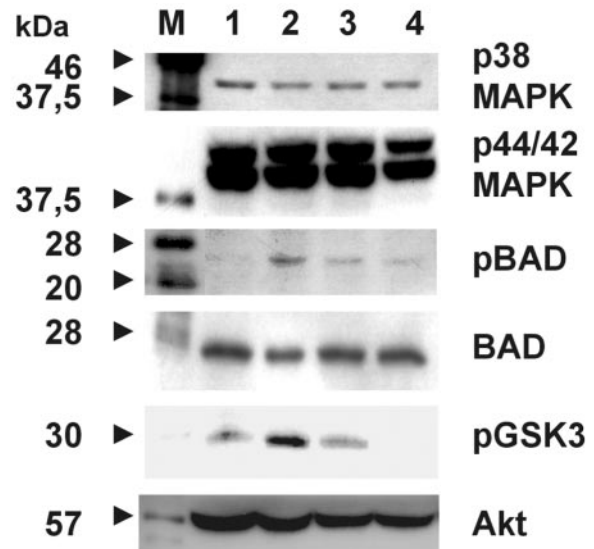
of LY294002 alone. LY294002 had no significant effect on neuronal viability, neither in OGD-treated (Fig. 9*A,B*) nor in untreated cortical neurons (data not shown).

The observation that EPO-induced neuroprotection is PI3K-dependent led us to test whether the activation of one of the major downstream effectors of PI3K, the serine-threonine protein kinase Akt, is correlated with the observed neuroprotection. Stimulation of cortical neurons with 100 U/l rhEPO for 30 min resulted in a marked activation of Akt, which was diminished significantly by sEpoR (Fig. 10). In addition, the activation of Akt induced by 100 U/l rhEPO was blocked completely in the presence of the specific PI3K inhibitor LY294002 (Fig. 10).

Bad has been identified as one major target of Akt, linking the PI3K pathway directly to the apoptotic machinery (Datta et al., 1999). We measured Bad phosphorylation by using a phospho-specific anti-Bad antibody that specifically recognizes the serine residue 136, because this residue has been shown to be a substrate of Akt kinase and thereby is sufficient to promote cell survival. As shown in Figure 10, a band corresponding to phosphorylated Bad was already present in untreated cultured neurons. However, after pretreatment of cortical neurons with 100 U/l EPO for 30 min, Bad phosphorylation increased significantly. EPO-induced Bad phosphorylation was diminished significantly after the blockade of EpoR by the application of sEpoR as well as after the specific inhibition of PI3K by LY294002 (Fig. 10). In all cases, Western blot analysis with a nonphospho-specific anti-Bad antibody showed that the protein levels of Bad were not altered by the treatment (Fig. 10), in accordance with our findings that EPO did not alter Bad transcription (Fig. 8). Furthermore, because Akt promotes survival in hippocampal neurons by preventing transcriptional activity of p53 and thereby blocking the induction of the proapoptotic Bcl-2 family member Bax (Yamaguchi et al., 2001), we tested whether EPO repressed Bax transcription. However, we found no repression of Bax transcription (Fig. 8).

## DISCUSSION

The key findings of our study were that (1) astrocytes as well as neurons can release paracrine signals that induce protection



**Figure 10.** Erythropoietin-induced neuroprotection is associated with the activation of Akt kinase and the phosphorylation of BAD. rhEPO (100 U/l) for 30 min activated Akt kinase and phosphorylated BAD, but not p38 and p44/42 MAPK, in cortical neurons. EPO-induced activation of Akt kinase and BAD phosphorylation is blocked by 2.5  $\mu$ g/ml sEpoR as well as by the specific PI3K inhibitor LY294002 (10  $\mu$ M). Shown are Western blot analysis from control cultures (lane 1), EPO-stimulated cultures (lane 2), sEpoR- and EPO-treated cultures (lane 3), and LY294002- and EPO-treated cultures (lane 4). The molecular sizes of the protein standards (lane M) are indicated (left). GSK3 indicates the substrate protein in the Akt kinase assay.

against hypoxia/ischemia in neurons; (2) a key mediator of paracrine neuroprotection by astrocytes (but not neurons) is EPO, which acts via its cognate receptor; (3) EPO-induced neuroprotection is effective within minutes and can be sustained for many hours with continued EPO exposure; (4) in neurons, EPO induces neuroprotection via a protein phosphorylation cascade including JAK-2 and PI3K/Akt that may inactivate the proapoptotic Bcl2 family member Bad; however, (5) other well described protective signaling pathways of EPO (STATs, ERK) did not seem to play a significant role in our system.

We have to consider that our *in vitro* model may be more relevant for neonatal ischemia, although the OGD model is a standard *in vitro* model of adult cerebral ischemia. In addition, we have experimental evidence that points to effects of EPO in the adult brain that are responsible for protection in ischemic tolerance. Specifically, sEpoR (intracerebroventricularly) attenuates hypoxic preconditioning against focal cerebral ischemia in mice (K. Prass, U. Dirnagl, and A. Meisel, unpublished data). We are thus confident that the *in vitro* evidence presented here is relevant for the mammalian adult brain *in vivo*.

Bernaudo et al. (2000) have shown that EPO expression is induced by hypoxia in neurons as well as in astrocytes. Furthermore, desferrioxamine, an activator of HIF-1 and potent stimulator of IP *in vitro* and *in vivo*, induced EPO mRNA in neurons and in astrocytes (Zaman et al., 1999; Bergeron et al., 2000; Bernaudo et al., 2000). However, results allowing a comparison of absolute EPO expression levels in astrocytes and neurons do not exist. Our results suggest that only astrocytes, but not neurons, express and release sufficient amounts of EPO for paracrine neuroprotection. Importantly, although neurons expressed and released IP-mediating factor(s), in accordance with our negative transcriptional data we were able to rule out by pharmacological



means (sEpoR and anti-EpoR) that EPO participates in inter-neuronal preconditioning. Liu et al. (2000) have identified a TNF- $\alpha$ -mediated and ceramide synthesis-dependent pathway of IP in an OGD model very similar to ours, a mechanism that has been corroborated recently by the same group *in vivo* (Furuya et al., 2001). Because TNF- $\alpha$  is expressed in an IP model *in vivo* (Wang et al., 2000), this cytokine should be considered as a promising candidate responsible for the paracrine protective signaling from neurons to neurons.

Pretreatment of cultured neurons with rhEPO (30 and 300 pM) 24 hr before exposure to NMDA led to a significant reduction of neuronal damage (Bernaudin et al., 1999). In “chronic” OGD models, with hypoxic intervals of up to 24 hr, rat cortical or hippocampal neurons (but not astrocytes) were protected in the presence of 30 pM recombinant mouse EPO or 100 pM rhEPO, respectively (Sinor and Greenberg, 2000; Sirén et al., 2001). Interestingly, in five patients with traumatic brain injuries Marti et al. (1997) reported EPO levels between 30 and 40 U/l in CSF. The EPO doses used in previous *in vitro* studies and in human CSF are therefore in good agreement with our data, in which 100 U/l rhEPO (corresponding to 30 pM) were effective. However, the design of these studies differed significantly from ours. First, our OGD stimulus was more severe, because 180 min of OGD was 100% lethal after 24 hr, whereas ~50% of neurons survived OGD periods of 24 hr in the model of Sinor and Greenberg (2000) and 15 hr in the model of Sirén et al. (2001). Second, these authors analyzed the survival of OGD-treated neurons in the presence of EPO during hypoxia. In contrast, we pretreated cortical neurons with EPO, which was washed out before OGD. Third, we demonstrated for the first time a neuroprotective effect of endogenous EPO *in vitro*. Therefore, our findings add to what was already known concerning EPO-mediated neuroprotection *in vitro*: EPO does not have to be present during OGD for protection; it also protects against severe OGD, and it can act in a paracrine manner.

We and others provide evidence that EPO protects neurons by preventing apoptosis (Digicaylioglu and Lipton, 2001; Sirén et al., 2001). We demonstrate that EPO-induced neuroprotection is EpoR-mediated. In erythropoiesis the EpoR signaling is mediated via a tyrosine phosphorylation cascade. In accordance with the functional role of JAK-2 in EpoR signaling in erythroid cells (Witthuhn et al., 1993), we found EPO-induced neuroprotection to be JAK-2-dependent. Our finding is in agreement with recent results by Digicaylioglu and Lipton (2001), who also provide strong evidence for an essential role of EpoR-mediated JAK-2 activation in neuroprotection.

The JAK-2/STAT pathway plays a key role in the anti-apoptotic signaling of EPO in hematopoiesis (Oda et al., 1998; Lawson et al., 2000). STAT1, STAT3, and STAT5 proteins have been implicated in the regulation of apoptosis (Bromberg, 2001). However, only STAT5 has been demonstrated in the anti-apoptotic signaling of EPO. In contrast to a large body of evidence on erythroid progenitors, we found no evidence for the induction of STAT5, STAT3, or STAT1 in EPO-induced neuroprotection. STATs exert their anti-apoptotic effects by the induction of *Bcl2* family proteins, in particular *Bcl-X<sub>L</sub>* (Silva et al., 1999; Socolovsky et al., 1999). We found no evidence for downstream transcriptional activation of the anti-apoptotic *Bcl/II* family genes *Bcl-X<sub>L</sub>*, *Bcl-2*, and *Bag-1*. We therefore conclude that, at least under the conditions studied here, the JAK/STAT pathway does not seem to play a significant role in EPO-induced neuroprotection.

In erythroid progenitor cells, EPO anti-apoptotic signaling is

mediated by ERK, a downstream-signaling kinase of the MAPK pathway (Sui et al., 2000). ERK activation correlates with neuroprotection in a rat model of global cerebral IP (Shamloo et al., 1999). In cultured rat hippocampal neurons ERK is activated by EPO under hypoxic conditions (Sirén et al., 2001). In contrast, we found no ERK activation in cortical cerebral neurons after EPO stimulation. The reasons for this discrepancy are unclear, but different anti-apoptotic pathways in hippocampal versus cortical cerebral neurons and other model differences (dosages, timing, etc.) have to be considered.

The ability of trophic factors to promote survival has been attributed, at least in part, to PI3K and its molecular targets (for review, see Datta et al., 1999). In particular, in erythroid precursors EPO prevents apoptotic cell death via a PI3K-dependent pathway (Bao et al., 1999; Lawson et al., 2000). In our experiments a specific inhibitor of PI3K (Davies et al., 2000) partially abolished EPO-induced protection against OGD. The fact that the inhibition was only partial may be a consequence of either incomplete inhibition of PI3K by LY294002 or the involvement of additional signaling pathways in EpoR-mediated neuroprotection (see below). The concentration of LY 294002 used in our study was comparable with that used in previous studies (Fruman et al., 1998) and could not be increased without the appearance of toxic side effects (K. Ruscher, unpublished observations). In agreement with our data, Sirén et al. (2001) have demonstrated a PI3K-dependent neuroprotective effect of EPO in rat hippocampal neurons. PI3K suppresses apoptotic cell death via its downstream effector protein kinase B (PKB)/Akt. Activated Akt kinase plays a central role in suppressing apoptosis by modulating the activities of *Bcl2* family proteins and caspase 9. PI3K specifically induces phosphorylation at Thr<sup>308</sup> and Ser<sup>473</sup>, which play a central role in Akt activation (Datta et al., 1999). We found EPO-induced and PI3K-mediated activation of Akt kinase activity in cultured cortical neurons. In accordance with this finding, Sirén et al. (2001) reported EPO-induced phosphorylation of Akt at Ser<sup>473</sup> by hypoxia in hippocampal neurons. Activated Akt, in turn, can phosphorylate Bad specifically at Ser<sup>136</sup> (del Peso et al., 1997) and thus can inactivate Bad. Phosphorylated Bad prevents apoptosis, because unphosphorylated Bad is capable of forming heterodimers with the anti-apoptotic proteins *Bcl-X<sub>L</sub>* or *Bcl-2* and thereby antagonizes their anti-apoptotic function (Zha et al., 1996). For the first time we describe a correlation between EPO-induced and PI3K-mediated phosphorylation of Bad at Ser<sup>136</sup> and neuroprotection. Our results suggest that Bad phosphorylation plays a role in EPO-induced prevention of neuronal apoptosis, but other PKB/Akt substrates like caspase 9 (Datta et al., 1999), also have to be considered.

Surprisingly, we found that even very short pretreatment (5 min) with EPO induces significant protection against OGD in neurons. Such a rapid effect is not consistent with the induction of protective gene expression by EPO. Post-translational regulation, for example protein phosphorylation, as described above, is more likely to account for such fast cellular responses. However, because in our experiments the neuroprotective effect is not only sustained but intensified after 24 hr of EPO exposure, the additional induction of gene expression programs seems likely. Recently, the activation of the transcription factor NF- $\kappa$ B has been reported as an essential step in EPO-induced neuroprotection (Digicaylioglu and Lipton, 2001). This pathway is JAK-2-dependent but PI3K-independent and thus may explain why in our study EPO-induced neuroprotection is blocked only partially by the specific PI3-kinase inhibitor LY294002, although this com-

pound inhibited Akt kinase completely and Bad phosphorylation nearly completely to control level. Remarkably, the activation of NF- $\kappa$ B is a key event for IP, as demonstrated recently in an *in vivo* model of global ischemia (Blondeau et al., 2001; Ravati et al., 2001).

In conclusion, our data provide evidence for a new, endogenous signaling cascade inducing tolerance against ischemia in the brain. Our results demonstrate that IP in the brain is the result of complex signaling programs involving various cellular elements of the brain. By a combination of transcriptional as well as post-translational mechanisms the brain can protect itself against the deprivation of substrate. Understanding these mechanisms may allow us in the future to induce or boost endogenous protection in patients as a novel strategy to safeguard the brain against hypoxia/ischemia.

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