Hypoxia-Induced Stroke Tolerance in the Mouse Is Mediated by Erythropoietin

Konstantin Prass, MD; Anna Scharff; Karsten Ruscher; Diana Löwl; Claudia Muselmann; Ilya Victorov, MD; Krisztian Kapinya, MD; Ulrich Dirnagl, MD; Andreas Meisel, MD

Background and Purpose—Cellular response to hypoxia is mainly controlled by hypoxia-inducible factor 1 (HIF-1). The HIF-1 target gene erythropoietin (EPO) has been described as neuroprotective. Thus, we hypothesize EPO to be an essential mediator of protection in hypoxic preconditioning.

Methods—We randomized Sv129 mice into groups for different pretreatments, different hypoxia-ischemia intervals, or different durations of ischemia. For hypoxic preconditioning, the animals were exposed to a hypoxic gas mixture (8% O2 and 92% N2) for 30, 60, 180, 300, or 360 minutes. At 0, 24, 48, 72, or 144 hours later, we performed middle cerebral artery occlusion and allowed reperfusion after 30, 45, 60, or 120 minutes, or occlusion was left to be permanent. We studied EPO gene expression in brain tissue with a real-time reverse transcriptase–polymerase chain reaction and measured HIF-1 DNA-binding activity with an electrophoretic mobility shift assay. To block endogenously produced EPO, we instilled soluble EPO receptor into the cerebral ventricle.

Results—Hypoxic preconditioning for 180 or 300 minutes induced relative tolerance to transient focal cerebral ischemia, as evidenced by a reduction of infarct volumes to 75% or 54% of the control, respectively. Hypoxic pretreatment was effective only when applied 48 or 72 hours before middle cerebral artery occlusion. Sixty minutes after hypoxia, we found a marked activation of HIF-1 DNA-binding activity and a 7-fold induction of EPO transcription. Infusion of soluble EPO receptor significantly reduced the protective effect of hypoxic pretreatment by 40%.

Conclusions—Endogenously produced EPO is an essential mediator of ischemic preconditioning. (Stroke. 2003;34:1981-1986.)

Key Words: erythropoietin ■ ischemic preconditioning ■ stroke ■ transcription factors ■ mice

It has long been known that the brain is very sensitive to hypoxia and ischemia. However, more recently it has been discovered that the brain, like other organs, is capable of inducing protective mechanisms when challenged by stresses or substrate deprivation. These mechanisms, collectively labeled as ischemic tolerance or ischemic preconditioning, are of putative importance for limiting the damage during substrate deprivation. Protection may occur within minutes, but this early tolerant state is lost within hours. However, tolerance can be seen in a second time window after 24 to 48 hours and then lasts for 2 to 3 days. Evidence for the existence of ischemic preconditioning in humans has been reported. The major goal of ischemic preconditioning research at present is to identify the underlying endogenous protective signaling cascades, with the long-term goal to allow therapeutic augmentation of the endogenous protective mechanisms in cerebral ischemia and possibly to induce a protected state of the brain in conditions in which brain ischemia can be anticipated, for example, during surgery of the heart or brain.

To study the mechanisms of ischemic preconditioning, robust and clinically relevant models are needed. Short and nondamaging periods of focal cerebral ischemia were reported to induce ischemic preconditioning in animals and in humans. Similarly, hypoxia has recently been identified to induce ischemic preconditioning in neonatal as well as in adult mice. To prevent oxygen deprivation or at least to adapt to it, all aerobic organisms are equipped with effective oxygen-sensing mechanisms. In this regard, the transcription factor hypoxia-inducible factor 1 (HIF-1) appears to be a universal molecular master switch, controlling cellular survival, glucose metabolism and transport, and metabolic adaptation. One of the most relevant target genes of HIF-1 is the glycoprotein hormone erythropoietin (EPO). Interestingly, it has been discovered (1) that EPO and its receptor EPO-R are expressed in the brain and in vivo and in vitro.

Since hypoxia induces HIF-1 in the brain and since HIF-1, in turn, induces the expression of the neuroprotectant EPO, it
seems straightforward to postulate a significant role for EPO in hypoxic preconditioning. In fact, in an in vitro model of ischemic preconditioning, EPO released by astrocytes acts as a paracrine mediator of neuroprotection. However, the evidence for a role of EPO in ischemic preconditioning, as proposed by Dawson, is merely correlational at present since no direct proof for an involvement of endogenously produced EPO in ischemic preconditioning in vivo is currently available.

In this study we establish and characterize a mouse model of hypoxic preconditioning with regard to stimulus dose, time window, and severity of ischemia. We further demonstrate that the preconditioning hypoxia induces HIF-1 DNA-binding activity and EPO expression in the brain, and we present for the first time functional evidence that EPO protein is an essential mediator of protection in hypoxic preconditioning.

Materials and Methods

Animal Model
For all experiments, we used mice (C3H/HeN, mixed sex, 18 to 22 g body wt). All experiments were performed and quantified in a randomized fashion by investigators blinded to the treatment groups. All surgical procedures were approved by the local authorities.

For hypoxic preconditioning, animals were transferred (maximum 6 mice per procedure) into an airtight, transparent chamber of 3000 mL volume at 21°C. A total gas flow of 400 mL/min of 92% N₂ and 8% O₂ (AGA) was established with the use of calibrated flowmeters (Cole Parmer), and partial oxygen pressure was measured intermittently by means of a polarographic electrode (Licox PO₂, GMS). For pretreatment, at least 3 mice per group underwent hypoxia (but not MCAO) as described above and were killed on the sixth day. Tissue preparation, at least 3 mice per group underwent hypoxia (but not MCAO) as described above and were killed on the sixth day.

MCAO was induced with an 8-0 nylon monofilament as described previously. Reperfusion was allowed after 30, 45, 60, or 120 minutes by removal of the monofilament under brief anesthesia. Occlusion remained permanent in 1 group. During surgery and 2 hours after reperfusion, body temperature was measured and maintained between 37.0°C and 37.5°C with a heating pad in all animals.

We infused recombinant soluble human EPO receptor (sEPO-R) into the cerebral ventricle as described. In brief, 2 hours after hypoxic preconditioning (or sham treatment), we anesthetized the mice, implanted an osmotic mini-pump (Alzet model 1003D, Alza) subcutaneously into the back, and then placed a needle from the pump into the left lateral ventricle. We infused sEPO-R (R&D Systems) in vehicle (PBS [pH 7.5] and 0.1% bovine serum albumin) at a dose of 6 μg/d in a volume of 1 μL/h and terminated infusion 6 hours before MCAO. Control animals received either vehicle at the same rate or heat-denatured sEPO-R (30 minutes at 60°C) at the same rate and dose.

Infarct sizes were determined 2 days after MCAO as described previously. To assess potential neuronal damage after hypoxic pretreatment, at least 3 mice per group underwent hypoxia (but not MCAO) as described above and were killed on the sixth day. Tissue processing followed the method described previously. We examined slides with light microscopy, giving special attention to hypoxia-vulnerable regions (eg, cerebral cortex and hippocampus).

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction of EPO mRNA
Total cellular RNA from neocortex was isolated, and RNA preparation and cDNA synthesis were performed as described. Expression of each sample was normalized for RNA preparation and reverse transcription (RT) reaction on the basis of its β-actin mRNA content. For detection of the amplification products in EPO reverse transcriptase–polymerase chain reaction (RT-PCR), we used EPO-specific internal hybridization probes. The LightCycler FastStart DNA Master Hybridization Probes Kit (Roche Molecular Biochemicals) was used as recommended by the manufacturer. Thermal cycling started with 10 minutes at 95°C and proceeded with 45 cycles of 95°C for 15 seconds, 64°C for 10 seconds, and 72°C for 15 seconds. For β-actin RT-PCR, we used the LightCycler FastStart DNA Master Kit (Roche Molecular Biochemicals) as recommended by the manufacturer. Thermal cycling started with 10 minutes at 95°C and proceeded with 30 cycles of 95°C for 15 seconds, 68°C for 10 seconds, and 72°C for 15 seconds (amplification product data acquisition at 86°C). For amplification and detection, we used LightCycler Relative Quantification Software (Roche Molecular Biochemicals). The following sequence-specific primers (Tibmolbiol) were used: β-actin forward: 5'-ACCCACACTGTGCCCATCTAA-3'; β-actin reverse: 5'-GCCACAGATTCTCACACCAA-3'; EPO forward: 5'-CTAGGCCTCACCTCAGCTCGG-3'; EPO reverse: 5'-GGAGCTCTGGAAGTTCTTTGGG-3'; EPO probe 1: 5'-LC-Red640-TAGACCAGGAAGCTTGACGAAATC-3'; EPO probe 2: 5'-CAGCTTCAGTTCCTCCCCGAGAAGTAGT-fluorescein-3'.

Fluorescent Electrophoretic Mobility Shift Assay
Fluorescent electrophoretic mobility shift assay was performed as described previously.

Erythropoietin Chemiluminescent Immunoassay
Tissue from mouse neocortex was homogenized, and EPO concentrations were measured by the EPO-Immulate assay according to the manufacturer’s instructions (DPC Biemann).

Statistical Analysis
Data are presented as mean±SD. For statistical comparisons of 2 groups, a 2-tailed Student’s t test was used; for >2 groups, a 1-way ANOVA with subsequent Bonferroni correction was applied. Values for P<.05 were considered statistically significant.

Results
During hypoxia, the animals displayed lethargy, reduced spontaneous movement, and tachypnea. The animals recovered (normal grooming behavior, exploration habits) within minutes after preconditioning. There were no differences in survival between the groups up to 5 hours of hypoxia. In the group pretreated for 6 hours, however, almost 60% of the mice died, some during hypoxia. Serious side effects, such as seizures, loss of body weight, and prolonged recovery, were observed only in this group.

Hypoxic preconditioning for 30 or 60 minutes did not protect against the noxious effect of 45 minutes of MCAO applied 3 days later. However, 3 or 5 hours of hypoxia were able to induce robust protection in experimental stroke (infarct volume reduced to 75% or 54% of control, respectively; Figure 1A), while longer periods of hypoxia were tested. During hypoxia, the animals displayed lethargy, reduced spontaneous movement, and tachypnea. The animals recovered (normal grooming behavior, exploration habits) within minutes after preconditioning. There were no differences in survival between the groups up to 5 hours of hypoxia. In the group pretreated for 6 hours, however, almost 60% of the mice died, some during hypoxia. Serious side effects, such as seizures, loss of body weight, and prolonged recovery, were observed only in this group.

Hypoxic preconditioning for 30 or 60 minutes did not protect against the noxious effect of 45 minutes of MCAO applied 3 days later. However, 3 or 5 hours of hypoxia were able to induce robust protection in experimental stroke (infarct volume reduced to 75% or 54% of control, respectively; Figure 1A), while longer periods of hypoxia (6 hours) in surviving animals did not induce protection (infarct volume +15%; Figure 1A).

Maximum protection after 5 hours of hypoxia (8% oxygen) was seen when 3 days were allowed to elapse between hypoxic preconditioning and MCAO (infarct volume reduction by 46%). A 2-day interval between hypoxia and MCAO also led to statistically significant protection (infarct volume reduction by 23%), while the 1- and 7-day intervals did not differ from the control (Figure 1B).

Hypoxic preconditioning (5 hours, with 3-day interval between preconditioning and MCAO) was protective in all tested durations of transient ischemia (infarct volumes: 30-minute MCAO, 38% reduction; 45-minute MCAO, 46% reduction; 60-minute MCAO, 43% reduction; 120-minute...
MCAO, 27% reduction) but failed to protect in permanent ischemia (Figure 1C).

Up to 3 hours of hypoxia at 8% oxygen did not cause any detectable damage at the light microscopic level in hippocampal or neocortical neurons when examined 6 days after hypoxia (for hippocampus, see Figure 2). After 5 hours of hypoxia, in some sections few pyknotic neurons were seen in the CA1 sector of the hippocampus but not in the neocortex or any other investigated brain structure. After 6 hours of hypoxia, severe damage to the CA1 sector became evident after 7 days (Figure 2).

As response to 5-hour hypoxic preconditioning, we demonstrated in the mouse neocortex a significant induction of EPO transcription and translation. Using a real-time RT-PCR approach, at 60 minutes after hypoxia we found a 6-fold increase of EPO, which returned to baseline 4 hours after hypoxia (Figure 3A). Using an EPO immunoassay, at 1 hour after preconditioning we found a strong induction of EPO protein expression (110 U/L), which was further increased 48 hours after preconditioning (236 U/L), whereas in a control mouse we could not detect any EPO protein (<1 U/L). Since EPO expression is controlled mainly by the transcription factor HIF-1, and HIF-1 appears to be of major importance in the signaling cascade of ischemic preconditioning, we studied whether activation of HIF-1 precedes EPO expression. Using fluorescent electrophoretic mobility shift assay, we observed a 4-fold induction of HIF-1 DNA-binding activity in nuclear extracts from the brains immediately after a 5-hour period of hypoxic preconditioning (Figure 3B and 3C).

We studied the functional role of EPO-mediated protection in hypoxic preconditioning by the intracerebroventricular infusion of recombinant sEPO-R as a scavenger for endogenously produced EPO. The results are summarized in Figure 4. Infusion of heat-inactivated sEPO-R, started 2 hours after hypoxia treatment and terminated 6 hours before MCAO, did not affect the infarct volume–reducing effect of hypoxic preconditioning (5 hours, with 3-day interval between preconditioning and MCAO). Denatured sEPO-R infusion 2 hours after sham preconditioning (normoxia in chamber) also failed to affect infarct volumes. In contrast, sEPO-R reduced the infarct reduction of hypoxic preconditioning by 38% (from 42% to 26%). An intracerebroventricular infusion of sEPO-R for 64 hours, which was terminated 6 hours before MCAO, as in all the aforementioned groups, had no effect on infarct volumes without hypoxic preconditioning. Physiological parameters were not significantly different between sEPO-R and heat-denatured sEPO-R animals (see the Table, which is available online at http://stroke.ahajournals.org).

**Discussion**

The major findings of our study were as follows: (1) Preexposure to hypoxia induces protection in focal cerebral ischemia. This protection reduces the volume of the resulting infarct size following cerebral ischemia.

**Figure 1.** Infarct volumes of control or hypoxically pretreated groups. A, Infarct volumes after different durations of hypoxia. Mice were pretreated for 30, 60, 180, 300, or 360 minutes with hypoxia. Three days later, MCAO was induced for 45 minutes. B, Infarct volumes after different pretreatment intervals. The x-axis represents different intervals (hours) between hypoxic preconditioning (5 hours) and MCAO (45 minutes). C, Infarct volumes after hypoxic preconditioning in different MCAO groups. The animals were hypoxically pretreated for 5 hours. Three days later, MCAO was induced for 30, 45, 60, or 120 minutes or permanently (perm.). Values are mean ± SD; n = animals per group. *Statistical significance between groups at P < 0.05 (ANOVA with Bonferroni correction for B, 2-tailed t test for A and C).

**Figure 2.** Vanadium acid fuchsin staining of hippocampal CA1 region after 1, 3, 5, or 6 hours of hypoxic pretreatment compared with untreated control animals. *Dead neuron.
ischemia. (2) A maximum of protection is seen 3 days after exposure to hypoxia. (3) Protection becomes most extensive when the preconditioning hypoxia is close to the threshold of hypoxic damage. (4) Preconditioning protects in various durations of transient focal cerebral ischemia but not in permanent ischemia. (5) Hypoxic preconditioning induces HIF-1 DNA-binding activity and subsequent EPO expression. (6) Scavenging of endogenous EPO reduces the protective effect of hypoxic preconditioning by approximately 40%, which provides strong evidence for an essential role of EPO in ischemic preconditioning.

In vitro, hypoxia is well known to induce protection. However, recently, hypoxic preconditioning has been more systematically characterized in neonatal and adult rodents. In our study hypoxic preconditioning induced tolerance only when reperfusion was allowed within 2 hours after transient focal cerebral ischemia, which is entirely in agreement with Miller et al. These authors exposed 3 different mouse strains to 2 hours of hypoxia (11% oxygen) and found 50% to 60% infarct volume reduction when 90 minutes of MCAO were induced 48 hours afterward. Our preconditioning paradigm (8% oxygen for 5 hours), which yielded a degree of protection similar to that reported by Miller et al, represents, however, a more severe hypoxia. Although the preconditioning stimulus in the study of Bernaudin et al is identical and the degree of protection is comparable to our model, there are some striking differences in the results: In contrast to our data and those of Miller et al, these authors demonstrate a significant infarct reduction in a mouse model of permanent focal cerebral ischemia. Furthermore, they can induce protection with 1 and 6 hours of hypoxia, in which we have not observed any infarct reduction. In our study 60% of the animals treated for 6 hours died, and the surviving animals showed severe damage in the CA1 sector. Our data do not necessarily imply that the hypoxic insult directly induced neuronal death, and other factors such as hypotension may have been of relevance.

Bernaudin et al reported a period of 24 hours between hypoxic preconditioning and ischemia to be sufficient for induction of tolerance, whereas in our study a period of 48 hours was necessary. Furthermore, we observed a maximal protection at 72 hours after preconditioning, a period in which Bernaudin et al could not demonstrate significant protection. It is unlikely that these discrepancies can find their explanation in the MCAO model, which was essentially identical in both studies. Since Bernaudin et al used Swiss mice and we worked with the Sv129 strain, we believe strain differences to be the more likely cause of the differing results. Significant strain differences in a preconditioning paradigm have been described recently.

Numerous mediators and mechanisms have been proposed to explain induced ischemic tolerance. Regarding hypoxia as
a preconditioning stimulus, it is straightforward to propose that protection, at least partially, is achieved via HIF-1-dependent signaling. HIF-1 is a key physiological sensor of the oxygen level in most mammalian cells. Hypoxia induces HIF-1, and HIF-1 then regulates the cellular transcriptional response to reduced oxygen availability to restore homeostasis of oxygen as a substrate for aerobic metabolism. In various models, activation of HIF-1 has been implicated as a key event in tolerance induction in vitro and in vivo. Among others, HIF-1 induces EPO. Only recently it was found that EPO is also expressed in the central nervous system and that it exerts potent neuroprotective effects (see Introduction). EPO transcription as well as EPO translation has been found in preconditioned brains. In addition, there is solid evidence that exogenously applied EPO is neuroprotective in vitro as well as in vivo. A phase I and II clinical trial suggests that EPO is not only safe but also beneficial in the therapy for acute stroke. EPO-mediated neuroprotection acts by antiapoptotic mechanisms and is mediated by Janus kinase-2–dependent pathways.

Recently, in an in vitro model of cerebral ischemia, strong evidence was provided that EPO expressed and released by astrocytes acts as a paracrine mediator for neuroprotection in ischemic preconditioning. Although a prominent functional role of endogenous EPO in ischemic preconditioning is plausible and likely, a proof for the mechanism had been missing. In this study we demonstrate such a function for EPO in ischemic preconditioning in vivo for the first time. Using exogenous application of a recombinant sEPO-R as a scavenger for endogenously produced EPO, we were able to significantly diminish tolerance induction by hypoxia preconditioning. By applying a similar approach, Sakana et al reported that EPO plays an important role in protecting neurons after cerebral ischemia. If endogenously produced EPO acts neuroprotectively, constitutive overexpression of EPO should result in smaller infarct size in models of focal cerebral ischemia. In a transgenic mouse line, with overexpression of human EPO preferentially in neuronal cells (4-fold elevated EPO levels in the brain), infarct volumes tended to be smaller (22%); however, this did not reach statistical significance compared with the control. Possible reasons for the missing significant neuroprotection in this mouse line may include the following: (1) Wiessner et al used a permanent model of focal cerebral ischemia, in which we also found no neuroprotection. (2) A 4-fold elevated EPO expression may be too low. (3) Whereas in the transgenic mice only EPO is overexpressed, in hypoxia preconditioned brains other potentially neuroprotective genes may also be induced. Thus, EPO may be necessary but not sufficient for neuroprotection. This is in accordance with the fact that we could not completely block tolerance induction with the sEPO-R. Although this may be explained by insufficient or incomplete binding of EPO by the scavenger, it appears more likely that there are also other protective signaling cascades involved in hypoxia-induced tolerance in the brain that are independent of EPO. Potential candidates include nitric oxide, adenosine, vascular endothelial growth factor, Bel-2, heat shock protein 70, and tumor necrosis factor-α.

In conclusion, we present for the first time strong experimental evidence for an essential functional role of endogenous EPO in ischemic preconditioning in vivo. Hypoxic preconditioning is an effective model for the ischemic tolerance phenomenon, in which further mediators of endogenous neuroprotection remain to be identified.

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