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.¹ However, more recently it has been discovered that the brain, like other organs, is capable of inducing protective mechanisms when challenged by stressors or substrate deprivation.² 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,³ 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.⁴ 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 animals⁵ and in humans.⁶ Similarly, hypoxia has recently been identified to induce ischemic preconditioning in neonatal⁷ as well as in adult mice.⁸ 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.⁹ 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 brain⁹-¹³ and (2) that the therapeutic administration of EPO can protect neurons against hypoxia-ischemia in vitro and in vivo.¹³-¹⁵ 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 (Sv129/J, BGVV-Berlin, 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 (Cole Parmer), and partial oxygen pressure was measured intermit-

- Middle cerebral artery occlusion (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), and partial oxygen pressure was measured intermittently by means of a polarographic electrode (Licox PO₂, GMS). For the control groups, room air was used at the same flow rate.

- Middle cerebral artery occlusion (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), and partial oxygen pressure was measured intermittently by means of a polarographic electrode (Licox PO₂, GMS). For the control groups, room air was used at the same flow rate.

- Middle cerebral artery occlusion (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), and partial oxygen pressure was measured intermittently by means of a polarographic electrode (Licox PO₂, GMS). For the control groups, room air was used at the same flow rate.

- Middle cerebral artery occlusion (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), and partial oxygen pressure was measured intermittently by means of a polarographic electrode (Licox PO₂, GMS). For the control groups, room air was used at the same flow rate.

- Middle cerebral artery occlusion (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), and partial oxygen pressure was measured intermittently by means of a polarographic electrode (Licox PO₂, GMS). For the control groups, room air was used at the same flow rate.

- Middle cerebral artery occlusion (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.

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<0.05 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 (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-1,8 and HIF-1 appears to be of major importance in the signaling cascade of ischemic preconditioning,6,16,18,21 we studied whether activation of HIF-1 precedes EPO expression. Using fluorescent electrophoretic mobility shift assay,16,20 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. (2) A maximum of protection is seen 3 days after exposure to hypoxia. (3) Prevention 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) 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 rodent models. Hypoxia is well known to induce protection. However, recently, hypoxic preconditioning has been more systematically characterized in neonatal and adult rodent models. Hypoxia is well known to induce protection. However, recently, hypoxic preconditioning has been more systematically characterized in neonatal and adult rodent models. Hypoxia is well known to induce protection. However, recently, hypoxic preconditioning has been more systematically characterized in neonatal and adult rodent models. Hypoxia is well known to induce protection. However, recently, hypoxic preconditioning has been more systematically characterized in neonatal and adult rodent models. Hypoxia is well known to induce protection. However, recently, hypoxic preconditioning has been more systematically characterized in neonatal and adult rodent models. Hypoxia is well known to induce protection. However, recently, hypoxic preconditioning has been more systematically characterized in neonatal and adult rodent models. Hypoxia is well known to induce protection. However, recently, hypoxic preconditioning has been more systematically characterized in neonatal and adult rodent models. Hypoxia is well known to induce protection. However, recently, hypoxic preconditioning has been more systematically characterized in neonatal and adult rodent models. Hypoxia is well known to induce protection. However, recently, hypoxic preconditioning has been more systematically characterized in neonatal and adult rodent models. Hypoxia is well known to induce protection. However, recently, hypoxic preconditioning has been more systematically characterized in neonatal and adult rodent models. Hypoxia is well known to induce protection. However, recently, hypoxic preconditioning has been more systematically characterized in neonatal and adult rodent models. Hypoxia is well known to induce protection. However, recently, hypoxic preconditioning has been more systematically characterized in neonatal and adult rodent models. Hypoxia is well known to induce protection. However, recently, hypoxic preconditioning has been more systematically characterized in neonatal and adult rodent models. Hypoxia is well known to induce protection. However, recently, hypoxic preconditioning has been more systematically characterized in neonatal and adult rodent models. Hypoxia is well known to induce protection. However, recently, hypoxic preconditioning has been more systematically characterized in neonatal and adult rodent models. Hypoxia is well known to induce protection. However, recently, hypoxic preconditioning has been more systematically characterized in neonatal and adult rodent models. Hypoxia is well known to induce protection. However, recently, hypoxic preconditioning has been more systematically characterize
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 anti-apoptotic mechanisms and is mediated by Janus kinase–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 hypoxic preconditioning. By applying a similar approach, Sakanaka 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 hypoxic-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.

**Acknowledgments**

This study was supported by the Hermann and Lilly Schilling Foundation and the Deutsche Forschungsgemeinschaft. The authors gratefully acknowledge the critical manuscript review by Tilo Wolf, MD.

**References**

Hypoxia-Induced Stroke Tolerance in the Mouse Is Mediated by Erythropoietin
Konstantin Prass, Anna Scharff, Karsten Ruscher, Diana Löwl, Claudia Muselmann, Ilya Victorov, Krisztian Kapinya, Ulrich Dirnagl and Andreas Meisel

*Stroke*. 2003;34:1981-1986; originally published online June 26, 2003;
doi: 10.1161/01.STR.0000080381.76409.B2

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/34/8/1981