Hypoxic preconditioning is an endogenous phenomenon in which sublethal hypoxic insult induces protection against future lethal hypoxia and other insults. Preconditioning exerts an endogenous protective mechanism that has been shown to help neurons survive further lethal insults. Although the mechanism involved in preconditioning is poorly understood, this conditioning has potential clinical prospects.

Hypoxia induces genes that have been shown to be associated with neuroprotection. One of the key genes involved is hypoxia-inducible factor-1 (HIF-1), a heterodimer consisting of HIF-1α and HIF-1β.1 Hypoxia induces an increase in HIF-1α by preventing its degradation in response to low O2 tension, whereas the HIF-1β subunit is constitutively expressed. HIF induces other downstream genes, one of which is erythropoietin (Epo), a glycoprotein that was demonstrated recently to have a neurological function by preventing neuronal apoptosis after cerebral ischemia2 and by protecting cultured cortical neurons from hypoxia3 and oxygen–glucose deprivation.4 Tolerance to hypoxia-induced stroke in mice is mediated by Epo.5

Hypoxia in neurons followed by reoxygenation involves the production of reactive oxygen species (ROS), which cause oxidative stress. Intracellular oxidative stress caused by ROS plays an important role in the mechanisms of hypoxic damage to brain neurons. Copper/zinc–superoxide dismutase (SOD1) scavenges superoxide radicals and protects cells from oxidative stress and ischemic injury.6,7 Increasing evidence points to the role of the redox signaling of oxygen radicals.8 The relationship among ROS, HIF-1, and Epo in oxidative stress signaling has not been examined. In the present study, using SOD1 transgenic (Tg) neuronal cultures, we investigated the role of Epo and ROS in hypoxic preconditioning and the related signaling pathways.

Materials and Methods

SOD1 Tg Mice
Heterozygous SOD1 Tg mice of the SOD1 TgHS/SF-218-3 strain carrying the human SOD1 gene with a 3-fold increase in SOD1 were derived from the founder stock described previously.9 There were no phenotypic differences between the SOD1 Tg mice and wild-type (Wt) littermates.
Cell Culture
Mouse cortical neurons were prepared from fetal cerebral hemispheres at gestational day 16 as described previously. Cultures were maintained in an incubator at 37°C and were used 8 to 10 days in vitro. For anoxic treatment, the cells were placed in a modular incubator chamber in humidified 95% N2/5% CO2 for 15 hours and then returned to the 37°C incubator. Preconditioning was performed 30 hours before anoxia, for 2 hours, in the same chamber. Antisense-Epo (25 nmol/L; 5'-CTCACCCGGGCACTTTCATG-3') and sense Epo (5'-ATGGGGGTGCCCCTGAGA-3'), as a control, were added directly to the cells, where required, before preconditioning.

Cell Death and Apoptosis Assay
Cell death was measured by modification of an immunohistochemical 2,3'-azino-bis(ethylbenzothiazoline-6-sulfonic acid) (ABTS) staining method with a neuron-specific antibody against mitogen-activated protein 2, which allowed for quantification of neurons using an ELISA plate reader at 405 nm, as described previously. For the apoptosis assay, a commercial enzyme immunoassay was used to determine cytoplasmic histone-associated DNA fragments.

Western Blot Analysis
Nuclear extracts were obtained using a nuclear extraction kit and following the manufacturer protocol (Sigma). Equal amounts of the samples were loaded per lane. The primary antibodies were HIF-1α (1:500; Novus Biologicals), Epo (1:200; Santa Cruz Biotechnology), caspase-3, pJAK2, pSTAT5, and nuclear factor-kB (NF-kB; 1:1000; Cell Signaling Technology). β-Actin (1:10 000; Sigma) and transcription factor IID (TFIID; Santa Cruz Biotechnology) were used as cytoplasmic and nuclear internal protein controls, respectively. Western blots were performed with horseradish peroxidase-conjugated immunoglobulin G (Cell Signaling Technology) with the use of enhanced chemiluminescence detection reagents (Amersham). Bands were scanned using a densitometer (GS-700; Bio-Rad Laboratories), and quantification was performed using Multi-Analyt 1.0.2 software (Bio-Rad).

RNA Isolation and RT-PCR Assays
Total RNA was prepared from cultured cells using a Micro-to-Midi Total RNA Purification system following the manufacturer protocol (Invitrogen). For RT-PCR analysis, a SuperScript 1-step RT-PCR kit with Platinum Taq (Invitrogen) was used following the manufacturer protocol. Mixtures were subjected to RT-PCR on a thermal cycler (Mastercycler Gradient; Eppendorf). Products were resolved on 1.5% agarose gels and were recorded with a gel documentation system (GS700; Bio-Rad).

Statistical Analysis
All data were from 3 separate experiments and were expressed as mean±SD. Comparisons among multiple groups were performed using a 1-way ANOVA with appropriate post hoc tests, whereas comparisons between 2 groups were achieved using Student’s t test (StatView 5.01; SAS Institute). P≤0.05 was considered statistically significant.

Results
Hypoxic Preconditioning Was Protective for Wt Neurons but not for SOD1 Tg Neurons
The ABTS/ELISA method was found to be a fast, reliable, and objective procedure for the quantification of neurotoxicity. In Wt neurons, 2 hours of hypoxic preconditioning 30 hours before severe hypoxia increased cell survival by 24% compared with neurons that underwent severe hypoxia alone. Although more SOD1 Tg neurons survived after 24 hours compared with Wt neurons, there was no significant difference in death after severe hypoxia with or without hypoxic preconditioning (Figure 1A). A cell apoptosis assay corroborated the neuronal quantification results, demonstrating that hypoxic preconditioning can reduce apoptosis after severe hypoxia by 2-fold (Figure 1B). Western blot analysis (Figure 2) showed that caspase-3 fragmentation, an apoptotic marker, was lower after preconditioned hypoxia compared with the hypoxia-only group. This supports the cell death assay results.

Protection by Hypoxic Preconditioning Mediated by Increased HIF-1α and Epo
RT-PCR results showed that levels of HIF-1α mRNA in mouse neuronal cultures before and after hypoxia and preconditioned hypoxia were unaltered. Western blots of the HIF-1α protein showed a significant increase after hypoxia compared with the control group and that it reached a higher level in neurons treated with 2 hours of preconditioning (Figure 3A). These results indicate that the hypoxic regulation of HIF-1α is post-translational and plays a role in neuroprotection after hypoxic insults. Epo, which is known to be regulated by HIF-1α activation, showed a remarkable increase after hypoxia and a further increase with preconditioned hypoxia at the transcription (mRNA) level as well as in the protein (Figure 3B). Densitometric analysis indicated that this increase in HIF-1α and Epo was higher in the preconditioned hypoxia group by 3- to 4-fold compared with the hypoxia-only group.
ROS Increased HIF-1α and Epo Expression in Neuronal Cultures After Hypoxia

Western blot analysis showed that although the levels of HIF-1α (Figure 4A) and Epo (Figure 4B) protein were elevated in Tg and Wt neurons after hypoxia, in the Tg neuronal culture, there was a significantly smaller increase compared with the Wt culture. This suggests that ROS are involved in HIF-1α and Epo expression. In the Wt neurons subjected to hypoxia, the HIF-1α protein disappeared within 1 hour after reoxygenation, whereas Epo was still increased 5 hours after hypoxia.

Epo Was a Critical Factor in Hypoxic Preconditioning and the Protective Effect of Epo Was Through Jak2–Stat5 and NF-κB

Western blot analysis showed an increase in pJak2 (Figure 5A), pStat5 (Figure 5B), and NF-κB (Figure 5C) immunostaining after hypoxia. In preconditioned hypoxia, this was further increased and accompanied by Epo protein expression. Administration of antisense Epo decreased the protective effect observed with preconditioning, whereas sense Epo did not have this effect. With the ABTS cell death assay (Figure 6A), we found that cell survival was reduced by 28% in the Wt neurons and by 13% in the Tg neurons compared with the sense control. Antisense Epo administration (Figure 6B) also increased cell apoptosis by 23% in the Wt neurons and by 11% in the Tg neurons compared with the sense control. These results suggest that Epo, to some extent, protects Tg neurons from hypoxic damage. Caspase-3 (Figure 6C) fragmentation increased with antisense Epo administration, indicating increased cell apoptosis. Western blot analysis demonstrated that Epo protein expression was blocked up to 90% after antisense Epo administration (Figure 6D). Administration of antisense Epo also resulted in a significant decrease in pJak2 (Figure 6E), pStat5 (Figure 6F), and NF-κB (Figure 6G) proteins compared with preconditioned hypoxia and the sense control group.

Figure 2. Western blot of caspase-3 after hypoxia (H) and preconditioned hypoxia (PH). The cleaved/active form of caspase-3 (19 kDa) was elevated in the hypoxic neurons compared with the preconditioned hypoxic cells and the control (C). Densitometric analysis showed a significant difference between the hypoxic and preconditioned hypoxic cells (*P<0.01). OD indicates optical density.

Figure 3. A, HIF-1α expression after hypoxia (H) and preconditioned hypoxia (PH). RT-PCR showed that there was no change at the mRNA level (hif-1α) between hypoxia and preconditioned hypoxia. Western blot analysis showed increased HIF-1α levels after hypoxia and preconditioned hypoxia. There was a significant increase in expression after preconditioned hypoxia relative to hypoxia only (*P<0.01). TFIID was used as an internal control. B, Epo expression after hypoxia (H) and preconditioned hypoxia (PH). An increase in Epo expression was seen in Epo mRNA (epo) and Epo protein after hypoxia, with a significant increase in preconditioned hypoxic neurons over hypoxic neurons. P indicates preconditioned neurons just before hypoxia; OD, optical density. β-Actin was used as an internal control. Numbers represent hours after hypoxia or preconditioned (precond.) hypoxia accordingly, and mRNA was measured at 1 hour.
Discussion

Hypoxia is known to be associated with an increase in ROS. Published studies support the idea that ROS affect ischemic central nervous system tissue through redox signal transduction pathways, in addition to the direct biochemical interaction between ROS and cellular macromolecules, which leads to cellular damage after cerebral ischemia.8,12 Other studies have shown that mild and short stimulation of cultured neurons with ROS generators made them less sensitive to subsequent insults,13 and that N-t-butyl-α-phenylnitrone, a free radical scavenger, abolished the protective action of hypoxic exposure.14 These reports indicate the importance of free radical production in protection against hypoxia. In the present study, overexpression of SOD1, a superoxide radical scavenger, ameliorated injury resulting from hypoxia, but it did not afford added protection after preconditioning. This suggests that ROS may be involved in hypoxic preconditioning.

Hypoxia is known to trigger HIF-1α,1 which can also be induced by cytokine-mediated ROS.15 The present study showed an upregulation of HIF-1α with hypoxia in the Wt neurons and a lower level of expression in the SOD1 Tg neuronal culture, although higher than in the untreated cells. Upregulation was observed at the protein level, whereas HIF-1α mRNA remained the same. This is consistent with other reports in which HIF-1α was post-translationally regulated by proline hydroxylation16 and allowed the neurons to react rapidly against hypoxia without the need for transcription or translation of a new protein. Induction of HIF-1α by ROS could occur when ROS bind to the iron in proline hydroxylase, thus inhibiting its activity. This suggests that the SOD1 Tg neuronal culture with less ROS would not inhibit proline hydroxylase activity, possibly resulting in a low level of HIF-1α expression.

With the induction of HIF-1α, other downstream genes, including Epo, are triggered. Epo was upregulated after hypoxia in preconditioned neurons as well as neurons treated with severe hypoxia only. In SOD1-overexpressing neurons, Epo was upregulated but at a much lower level compared with its activation in Wt neurons. Chandel et al17 reported that cells that were depleted of mitochondrial DNA (p0 cells) failed to increase ROS generation during hypoxia and failed to activate Epo mRNA. We have shown for the first time that SOD1 influences the upregulation of the Epo protein, thus demonstrating that ROS are an important factor in upregulation of Epo. After hypoxia with 2 hours of preconditioning, expression of HIF-1α and Epo was significantly increased and was higher than after lethal hypoxia. It is noteworthy that 30 hours after preconditioning (ie, just before severe hypoxia), increased Epo expression was not observed. This is consistent with other studies that reported that the sublethal preconditioning stimulus is not potent enough to induce
substantial concentrations of Epo. These neurons, when further treated with hypoxia, showed a significant increase in the Epo protein level over nonpreconditioned hypoxic neurons. This suggests that the threshold for the induction of HIF-1α/HIF-2α and Epo was lowered by preconditioning. Administration of antisense Epo before the preconditioning stimulus blocked neuroprotection against hypoxic stress. Our study shows for the first time that endogenous Epo plays a role in neuroprotection in vitro.

The mechanism by which Epo provides neuroprotection is not clearly understood. Epo combines with its receptor, Epo-R, and dimerization of the receptor activates Jak2 by phosphorylation. Jak2 phosphorylation is reported to activate phosphorylation of other kinases such as phosphatidylinositol 3-kinase-Akt/protein kinase B, Ras-mitogen-activated protein kinase, and the transcription factor Stat family. There is emerging evidence that the Jak–Stat pathway has an essential role in the development of the cardioprotected phenotype associated with ischemic preconditioning. Previous studies indicated an antiapoptotic role for Jak–Stat signaling. Stat5 proteins were shown to mediate the induction of the Bcl-xL gene, which is regarded as a potential regulator of cytokine-induced survival signals. An Epo-responsive motif for the binding of a Stat protein has been identified in the untranslated 5′ region of the mouse bcl-x gene. Recently, an alternate mechanism has been described by which Epo triggers cross-talk between the signaling pathways of Jak2 and NF-κB, another transcription factor that can enter the nuclei and activate the genes involved in neuroprotection. NF-κB activation induces overexpression of Bcl-2 and Bcl-xL, which function to prevent apoptotic cell death. The present study demonstrates significant increases in Jak2, Stat5, and NF-κB after preconditioning. Blockade of Epo by antisense Epo administration resulted in a significant reduction in cell survival, suggesting a role for Epo in neuroprotection. Furthermore, the downstream signaling molecules pJak2, pStat5, and NF-κB were also decreased, indicating neuroprotection occurs through the Jak2–Stat and NF-κB pathways.

A proposed mechanism for neuroprotection by preconditioning may involve sublethal ROS production, which does not damage the neurons, yet may be enough to act as a signal to downregulate the threshold for expression of HIF-1α and Epo proteins on the subsequent lethal hypoxia. As we have shown here, HIF-1α and Epo were activated in the preconditioned neurons and reached a higher level than in hypoxia without preconditioning. Epo combines with its receptor and activates Jak2. This would then trigger the downstream...
proteins, Stat5 and NF-κB, which are known to be transcription factors. This correlates with reports suggesting the presence of a time window for hypoxic preconditioning that ranges from 1 day to a few days. If the time between preconditioning and insult is too short, there is no time for the cells to recover, and continued hypoxia becomes lethal; if it is too long, the protective effect is lost because of a return to the threshold of nonpreconditioned cells.

Summary

Our study suggests that ROS induced by preconditioning may downregulate the threshold for HIF-1α and Epo activation, which plays a significant role in stimulating neuroprotection through the Jak–Stat and NF-κB pathways.

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Jing Liu, Purnima Narasimhan, Fengshan Yu and Pak H. Chan

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