

# Role of Hypoxia-Inducible Factor-1 in Hypoxia-Induced Ischemic Tolerance in Neonatal Rat Brain

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Hypoxia-inducible factor-1 (HIF-1) is a heterodimer composed of HIF-1 $\alpha$  and HIF-1 $\beta$  protein subunits. This transcription factor is essential for the activation of hypoxia-inducible genes like erythropoietin, some glucose transporters, the glycolytic enzymes, and vascular endothelial growth factor. Because HIF-1 activation may promote cell survival in hypoxic tissues, we studied the effect of hypoxic preconditioning on HIF-1 expression in neonatal rat brain. Hypoxic preconditioning (8% O<sub>2</sub> for 3 hours), a treatment known to protect the newborn rat brain against hypoxic-ischemic injury, markedly increased HIF-1 $\alpha$  and HIF-1 $\beta$  expression. To support the role of HIF-1 in protective preconditioning, we also studied the effect of two other known HIF-1 inducers, cobalt chloride (CoCl<sub>2</sub>) and desferrioxamine (DFX), on HIF-1 expression and neuroprotection in newborn brain. HIF-1 $\alpha$  and HIF-1 $\beta$  protein levels were markedly increased after intraperitoneal injection of CoCl<sub>2</sub> (60 mg/kg) and moderately increased after intraperitoneal injection of DFX (200 mg/kg) 1 to 3 hours after the injections. Preconditioning with CoCl<sub>2</sub> or DFX 24 hours before hypoxia-ischemia afforded 75 and 56% brain protection, respectively, compared with that in vehicle-injected littermate controls. Thus, HIF-1 activation could contribute to protective brain preconditioning, which could be used in high-risk deliveries and other clinical situations.

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Preconditioning is a process by which a tissue is rendered more tolerant to a subsequent lethal insult such as ischemia. Tolerance is attained by subjecting tissues to a sublethal stress. This is believed to produce intracellular adaptations that enhance endogenous defense mechanisms. In brain, neuronal tolerance against ischemic injury has been reported after preconditioning with a brief sublethal episode of ischemia,<sup>1</sup> spreading depression,<sup>2</sup> or exposure to metabolic inhibitors.<sup>3</sup> In addition, hypoxia preconditioning has been shown to produce tolerance against hypoxic-ischemic brain injury in newborn rats<sup>4–6</sup> and against kainic acid-induced brain lesions in adult rats.<sup>7</sup> Induced tolerance has also been observed in rat hippocampal slices preconditioned by anoxia<sup>8</sup> and in primary neuronal cultures pre-exposed to oxygen-glucose deprivation.<sup>9</sup> Interestingly, despite several studies on hypoxia-induced tolerance in rodent brain, the mechanisms underlying this neuroprotection remain poorly understood. Because it generally takes several hours to days after the preconditioning episode before a state of tolerance is

attained, the preconditioning stimulus is likely to involve adaptive changes in gene expression.<sup>1</sup> Accordingly, using a reproducible model of hypoxia-induced tolerance in the immature rat,<sup>4–6</sup> the present study was undertaken to elucidate the molecular mechanisms underlying this hypoxic preconditioning.

Hypoxia-inducible factor-1 (HIF-1), a basic helix-loop-helix PAS transcription factor, plays a central role in the cellular regulation of a broad variety of hypoxia-inducible genes.<sup>10</sup> HIF-1 binds to a specific DNA sequence in enhancers and promoters of many target genes such as erythropoietin, some glucose transporters, several glycolytic enzymes, and vascular endothelial growth factor (VEGF).<sup>10,11</sup> HIF-1 is a heterodimer made of two protein subunits, HIF-1 $\alpha$  and HIF-1 $\beta$  (identical to ARNT or aryl hydrocarbon nuclear receptor translocator).<sup>12</sup> HIF-1 $\alpha$  is unique to the HIF-1 heterodimer, whereas HIF-1 $\beta$  is involved in several other cellular functions through heterodimerization with other proteins such as the aryl hydrocarbon receptor,<sup>13</sup> HIF-2 $\alpha$ ,<sup>14,15</sup> or the recently cloned HIF-3 $\alpha$ .<sup>16</sup>

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HIF-1 $\alpha$  shows a high affinity for HIF-1 $\beta$  and competes with the aryl hydrocarbon receptor for the recruitment of HIF-1 $\beta$ .<sup>17</sup> Heterodimerization of HIF-1 $\alpha$  with HIF-1 $\beta$  is necessary for stable association within the nuclear compartment and for resistance to proteolytic degradation by the ubiquitin-proteasome system.<sup>18,19</sup> In addition, induction of HIF-1 DNA binding activity can be inhibited by pretreatment of hypoxic cells with actinomycin-D or cycloheximide, thus suggesting that ongoing RNA transcription and protein synthesis may be required for HIF-1 functional activation.<sup>20,21</sup>

HIF-1 expression is tightly regulated by oxygen availability. HIF-1 $\alpha$  expression and HIF-1 DNA binding activity increase exponentially as cellular oxygen concentrations decrease.<sup>22</sup> Thus, HIF-1 $\alpha$  protein accumulates during hypoxia but is rapidly degraded by the proteasome system on reoxygenation.<sup>12,19,23,24</sup> In contrast, HIF-1 $\beta$  expression is modestly affected by a lack of oxygen. Chemical hypoxia produced by inhibitors of mitochondrial respiration does not stimulate HIF-1 activity,<sup>11,22</sup> suggesting that HIF-1 activation is dependent on the oxygen concentration in the cellular environment. In addition to hypoxia, the divalent metal cobalt chloride (CoCl<sub>2</sub>) and the iron chelating agent desferrioxamine (DFX) stimulate HIF-1 expression, HIF-1 DNA binding activity, and the transactivation of several HIF-1 target genes under normoxic conditions.<sup>25–27</sup> The mechanism of action of these compounds is still unclear, but it seems to relate to a ferroprotein oxygen sensor.<sup>25,28</sup>

Because activation of HIF-1 may promote a widespread adaptive response in hypoxic cells by increasing glucose transport, glycolysis, vascular growth, and cell survival, we studied the effect of hypoxic preconditioning<sup>4</sup> on HIF-1 expression in newborn rat brain. In addition, because CoCl<sub>2</sub> and DFX are strong inducers of HIF-1 activity *in vitro*,<sup>25–27</sup> we investigated the effect of CoCl<sub>2</sub> and DFX pretreatment on neuroprotection and HIF-1 expression in the immature brain. Some of these results have been presented previously in abstract form.<sup>29</sup>

## Patients and Methods

### Animal Protocols

All experiments were performed in strict accordance with the relevant National Institutes of Health guidelines. All protocols were approved by the Committee on Animal Research at the University of California in San Francisco and at Washington University in St Louis. Hypoxic preconditioning was performed as previously described.<sup>4–6,30</sup> Briefly, male and female Sprague-Dawley rats ( $n = 22$ ) at postnatal day (P) 6 were placed in an 8% O<sub>2</sub> and 92% N<sub>2</sub> humidified atmosphere in 500-ml chambers partially submerged in a water bath maintained at a constant temperature of 37°C for 3 hours. This treatment has been shown to protect the newborn brain from subsequent hypoxic-ischemic injury.<sup>4–6</sup>

Control littermates ( $n = 21$ ) were maintained at 37°C in normoxic humidified conditions for 3 hours. For Western and Northern blot analyses of HIF-1 expression, brains from hypoxic rat pups ( $n = 8$ ) and normoxic control littermates ( $n = 8$ ) were quickly removed, dissected on ice, and frozen at –70°C immediately after the end of treatment. For immunohistochemistry, brains from some preconditioned ( $n = 4$ ) and control normoxic ( $n = 3$ ) animals were perfusion-fixed with 4% paraformaldehyde at the end of treatment.

To establish the role of HIF-1 in ischemic tolerance, we also studied the effect of known HIF-1 inducers other than hypoxia. Because it has been shown that HIF-1 is strongly activated *in vitro* by exposure to DFX or CoCl<sub>2</sub>,<sup>25–27</sup> separate groups of P6 rat pups were administered a single intraperitoneal injection of either DFX solution (200 mg/kg;  $n = 36$ ) or CoCl<sub>2</sub> solution (60 mg/kg;  $n = 35$ ). Control littermates ( $n = 30$  and  $n = 27$ , respectively) were injected with 0.9% sodium chloride vehicle solution (pH 7.4). Blot protein analysis of HIF-1 expression was performed on some brains at either 1 ( $n = 3$ ,  $n = 3$ , or  $n = 2$ ), 3 ( $n = 7$ ,  $n = 4$ , or  $n = 2$ ), or 6 hours ( $n = 3$ ,  $n = 2$ , or  $n = 2$ ) after DFX, CoCl<sub>2</sub>, or vehicle administration, respectively.

We then investigated whether DFX and CoCl<sub>2</sub> preconditioning in rat pups produced brain protection similar to that observed after hypoxic preconditioning.<sup>4–6</sup> Twenty-four hours after hypoxic or chemical preconditioning, the remaining rat pups pretreated with either hypoxia ( $n = 10$ ), DFX ( $n = 23$ ), or CoCl<sub>2</sub> ( $n = 26$ ) along with their littermate controls ( $n = 10$ ,  $n = 27$ ,  $n = 24$ , respectively) were anesthetized with a gas mixture containing 1% halothane in 30% O<sub>2</sub> and 70% N<sub>2</sub>O. The left common carotid artery was permanently ligated through a ventral midline neck incision as described previously.<sup>4–6,30,31</sup> The wound was sutured, and the pups were returned to their dam for 1.5 hours. The pups were then placed in an 8% O<sub>2</sub> and 92% N<sub>2</sub> humidified atmosphere at a constant temperature of 37°C for 3 hours. In healthy rat pups at P7, this procedure is known to produce selective brain damage or infarction in the hemisphere ipsilateral to the carotid occlusion, whereas hypoxia alone (contralateral hemisphere) does not result in brain damage.<sup>4–6,30–32</sup> After hypoxia-ischemia, all animals were returned to their dams. One week after hypoxic-ischemic injury, pups (at P14) were anesthetized with halothane, and their brains were removed, placed in chilled saline solution, and processed as detailed previously.<sup>4,5</sup> Briefly, after removal of the brainstem and cerebellum, the forebrain was sectioned at the midline, and left and right hemispheric weights were determined on a microbalance. Hemispheric necrosis was reflected by a reduction in hemispheric weight ipsilateral to the carotid ligation, which was calculated as the ratio of left to right hemispheric weights. Brains were then frozen on dry ice, sectioned on a cryostat, and Nissl-stained with cresyl violet. Because Nissl staining has been shown to closely correlate with the degree of brain necrosis assessed by tissue weight,<sup>4,5</sup> only tissue weight data are shown in the present study. In addition, because the neuroprotective effect of hypoxic preconditioning found in the present study was similar to that of previous reports,<sup>4–6</sup> only the data from DFX and CoCl<sub>2</sub> preconditioning are shown.

### Northern Blot Analysis

Immediately at the end of hypoxic preconditioning or hypoxia-ischemia treatments (before reoxygenation), rat pups were anesthetized with an intraperitoneal injection (0.3 g/kg) of pentobarbital sodium (Nembutal) and decapitated, and their brains were removed. The cerebral cortex between interaural planes 5.9 and 0.0 mm was quickly dissected<sup>33</sup> from each hemisphere and frozen separately on dry ice. Brains from normoxic control rat pups were processed in a similar fashion. For each treatment, messenger RNA (mRNA) extracts were prepared using the FasTrack 2.0 Kit (Invitrogen, San Diego, CA) according to the manufacturer's protocol. After quantitation by ultraviolet absorption at 260 nm, aliquots of mRNA (10 µg) were separated by denaturing gel electrophoresis (formaldehyde and 1% agarose). After separation, the mRNA was transferred overnight to nylon membranes (Hybond-N<sup>+</sup>; Amersham, Arlington Heights, IL) by upward capillary action (TurboBlotter; Hoefer-Pharmacia, San Francisco, CA) using 20× sodium chloride and sodium citrate solution. The membranes were then ultraviolet-crosslinked and incubated at 42°C for at least 4 hours in prehybridization buffer containing 6 × sodium chloride and sodium citrate solution, 2 × Denhardt's solution, 1% sarcosyl, 10% dextran sulfate, 50 mM of phosphate buffer (BP) (pH 7.0), and 250 µg/ml of heat-denatured salmon sperm DNA. An anti-sense oligonucleotide probe complementary to the mRNA encoding the mouse HIF-1α (5'-CGTTGCTGACCTTGATGTTTCATCGTCCTCCCCGGCTTGTAGGG-3', bases 788–831; Genbank accession number U59496)<sup>34</sup> was labeled at the 3'-end with <sup>32</sup>P-deoxyadenosine triphosphate (DuPont-NEN Research Products, Boston, MA) using terminal deoxynucleotidyltransferase (Gibco-BRL, Gaithersburg, MD). To remove unincorporated labeling, the reaction mixture was passed through an STE Micro Select-D G-25 spin column (5 Prime–3 Prime, Boulder, CO). Hybridization was then carried out at 42°C for 12 to 18 hours by adding 1.5 × 10<sup>6</sup> cpm/ml of <sup>32</sup>P-labeled probe. After hybridization, the blots were washed extensively as follows: twice with 2 × sodium chloride, sodium phosphate, and EDTA solution (SSPE) with 0.1% sodium dodecyl sulfate (SDS) at 42°C for 30 minutes; once with 0.5 × SSPE and 0.1% SDS at 37°C for 30 minutes; and once with 0.2 × SSPE and 0.1% SDS at 37°C for 30 minutes. Membranes were then exposed to Kodak XAR-2 film (Rochester, NY) at –70°C for up to 1 week using one intensifying screen. The membranes were stripped and reprobed with a labeled anti-sense oligonucleotide complementary to the mRNA encoding the rat HIF-1β (5'-CCTGCTGGTGGCCAGGCCTTGATGTAACCTGTGCAGTGGACTAC-3', bases 628–671; Genbank accession number U08986)<sup>35</sup> following the same procedure described previously. A computer-based imaging system (MCID; Imaging Research, St Catharines, Ontario, Canada) was used to measure the band area and density of each transcript on the autoradiograms after subtraction of the film background.

### Immunoblot Analysis

Control rat pups and rats subjected to either hypoxic preconditioning, unilateral hypoxia-ischemia, or DFX or CoCl<sub>2</sub> injection were deeply anesthetized. After decapitation, brains

were quickly removed, and the cerebral hemispheres (between interaural planes 5.9 mm and 0.0 mm)<sup>33</sup> were separated and frozen separately at –70°C until analysis. Sample preparation and analysis were performed as described previously<sup>36,37</sup> with minor modifications. Brain tissue was homogenized in 20 mM of HEPES (pH 7.5), 1.5 mM of MgCl<sub>2</sub>, 0.2 mM of EDTA, 100 mM of sodium chloride, 2 mM of dithiothreitol, 0.4 mM of phenylmethylsulfonyl fluoride, and 1 mM of sodium orthovanadate. Sodium chloride (4 M) was added to a final concentration of 0.45 M. The homogenate was then centrifuged 30 minutes at 10,000g, and the supernatant was mixed with an equal volume of 20 mM of HEPES (pH 7.5), 1.5 mM of MgCl<sub>2</sub>, 0.2 mM of EDTA, 0.45 mM of sodium chloride, 40% (vol/vol) of glycerol, 2 mM of dithiothreitol, 0.4 mM of phenylmethylsulfonyl fluoride, and 1 mM of sodium orthovanadate. Protein concentrations were determined using the Bradford method (BioRad, Richmond, CA). Equal protein samples (150 µg) were separated on 7% SDS polyacrylamide gels with 4.5% SDS stacking gels (SDS-polyacrylamide gel electrophoresis) and electrotransferred onto nitrocellulose membranes (0.2 µm; Schleicher and Schuell, Keene, NH). The membranes were blocked in 5% (wt/vol) nonfat dry milk in a solution of 20 mM of Tris-HCl (pH 7.6), 137 mM of sodium chloride, and 0.1% Tween-20 (TBS-T). Blots were then washed with TBS-T and incubated for 2 hours with an affinity-purified HIF-1α antibody diluted at a ratio of 1:500. This rabbit polyclonal antibody was raised against recombinant proteins in which glutathione-S-transferase was fused to amino acids 329 through 531 of human HIF-1α.<sup>26</sup> After washes, the membranes were incubated with a 1:5000 dilution of anti-rabbit immunoglobulin-horseradish peroxidase antibody (Amersham) for 1 hour, and bound antibody was visualized with the enhanced chemiluminescence system (Amersham). To visualize HIF-1β protein on the same blot, HIF-1α antibody and enhanced chemiluminescence reagents were washed off the nitrocellulose membranes by incubation in TBS-T at 55°C for 30 minutes. The membranes were then reprobed with HIF-1β antiserum diluted at a ratio of 1:2000. This rabbit polyclonal antibody was raised against amino acids 496 through 789 of human HIF-1β/ARNT.<sup>26</sup> Subsequent washes and visualization procedures were as described for HIF-1α antibody. The MCID imaging system (Imaging Research) was used to measure the densities and areas of HIF-1α and HIF-1β protein signals on radiographic films. The relative density of HIF-1α (~120 kd) and HIF-1β (~91–94 kd) protein bands was analyzed after subtraction of the film background.

### Immunohistochemistry

Rats were deeply anesthetized as described previously and perfused through the left ventricle (via the ascending aorta) with 0.9% sodium chloride solution followed by 4% paraformaldehyde made up in 0.1 M of PB, pH 7.4. Brains were removed, postfixed in 4% paraformaldehyde for no more than 4 hours, and stored in 30% sucrose overnight at 4°C. Fifty-micrometer thick coronal sections were cut on a vibratome and washed twice with 0.05 M of PB, pH 7.4. After inhibition of endogenous peroxidase in 0.05 M of PB, pH 7.4, containing 0.65% sodium azide and 0.2% hydrogen



peroxide, sections were incubated for 1 to 2 hours in 0.1 M of PB containing 5% nonfat dry milk, 1% bovine serum albumin, 2% goat serum, 0.1% Triton X-100, and 0.1% rat serum. Sections were then incubated overnight at 4°C with the HIF-1 $\alpha$  antibody diluted at a ratio of 1:500 in 0.1 M of PB containing 1% bovine serum albumin, 2% goat serum, and 0.1% Triton X-100. Negative controls included alternate sections from each brain incubated without the primary antibody. After 3 washes in 0.05 M of PB, sections were incubated at room temperature for 2 hours with a 1:200 dilution of biotinylated goat anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA). Sections were then incubated in an avidin-horseradish peroxidase solution (Elite Vectastain; Vector Laboratories) for 2 hours, followed by washes with 0.05 M of PB. HIF-1 $\alpha$  immunostaining was visualized by incubating the sections with 0.015% 3,3-diaminobenzidine tetrachloride (Sigma, St Louis, MO) and 0.01% H<sub>2</sub>O<sub>2</sub> made up in 0.05 M of PB. After washing, sections were mounted on gelatin-coated slides, dried, and cover-slipped.

#### Data Analysis

The degree of hypoxic-ischemic brain injury after DFX or CoCl<sub>2</sub> pretreatment was expressed as the percentage of reduction in tissue weight of the hemisphere ipsilateral to the carotid ligation. This is calculated as a ratio of left (ipsilateral, ischemic) to right (contralateral, nonischemic) hemispheric weights as described previously.<sup>4,5</sup> Histograms represent the mean  $\pm$  SD for each distribution. Differences between vehicle- and drug-treated groups were tested by the unpaired, two-tailed, Mann-Whitney nonparametric test. A probability value less than 0.05 was considered significant.

## Results

### HIF-1 Is Constitutively Expressed in Newborn Rat Brain

Northern blot analysis of newborn rat brain demonstrated that the anti-sense oligonucleotides used for hybridization recognized a single band of the appropriate size for the HIF-1 $\alpha$  and HIF-1 $\beta$  mRNA transcripts (4.2 and 3.7 kb, respectively) (Fig 1). HIF-1 $\alpha$  and HIF-1 $\beta$  mRNA was constitutively expressed at a low but detectable level (see Fig 1, lane 1), consistent with previous studies in normal adult rat and human brain<sup>37,38</sup> as well as in embryonic mouse brain.<sup>39</sup> Similarly, a low level of HIF-1 $\alpha$  and HIF-1 $\beta$  proteins were detected in control brain tissue by Western blot analysis (Fig 2, lane 1). Immunohistochemistry confirmed that there is constitutive HIF-1 $\alpha$  expression throughout the newborn rat brain, with intense staining in regions enriched with neuronal cell bodies, including the molecular layer of CA1 through CA3 subfields of the hippocampus (Fig 3B) and in layers 1 to 3 of the cerebral cortex (see Figs 3A and 4A). Moderate HIF-1 $\alpha$  immunostaining was also noted in the striatum (see Fig 4D), the ventral thalamic nucleus, and the periventricular and ventromedial hypothalamic nuclei (not shown).

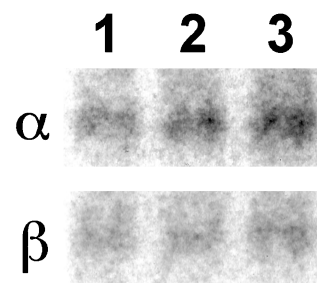


Fig 1. Northern blot analysis of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) and HIF-1 $\beta$  messenger RNA (mRNA) in the cerebral cortex of newborn rats subjected to hypoxic preconditioning or hypoxia-ischemia. Anti-sense oligonucleotide probes complementary to the mRNA encoding the mouse HIF-1 $\alpha$  or the rat HIF-1 $\beta$  show that they each identify single transcripts of approximately 3.7 and 4.2 kb, respectively. Lane 1 = untreated control, lane 2 = hypoxia, lane 3 = hypoxia-ischemia.

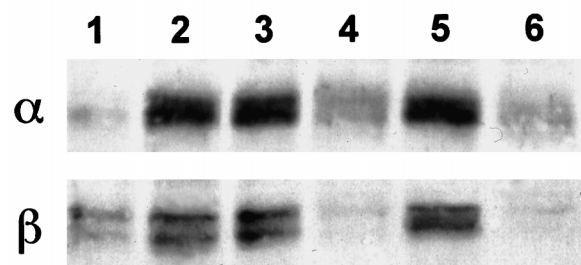
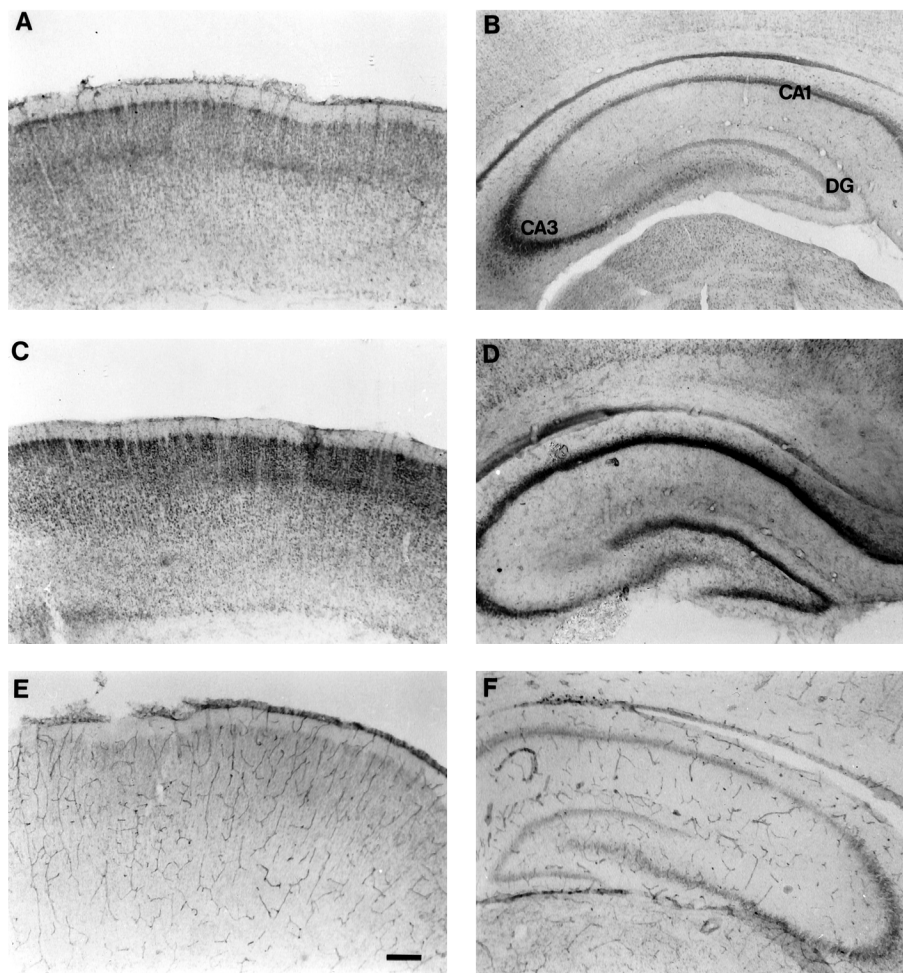


Fig 2. Western blot analysis of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) and HIF-1 $\beta$  proteins in the cerebral cortex of newborn rats subjected to hypoxic preconditioning or hypoxia-ischemia. Brains were analyzed at the end of each treatment before reoxygenation. Equal amount of protein samples (150  $\mu$ g) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with rabbit anti-HIF-1 $\alpha$  and anti-HIF-1 $\beta$  polyclonal antibodies. Lane 1 = untreated control, lane 2 = hypoxic preconditioning (8% O<sub>2</sub> for 3 hours), lanes 3–6 = contralateral (lanes 3 and 5) and ipsilateral (lanes 4 and 6) cortices from 2 different rat pups subjected to hypoxia-ischemia. The contralateral brain tissue was exposed to hypoxia without the common carotid artery ligation, whereas the ipsilateral brain tissue was ischemic secondary to the combination of carotid artery ligation and concurrent exposure to hypoxia.

As reported previously,<sup>30</sup> negative controls showed no endogenous staining throughout the brain (not shown).

### Hypoxia Preconditioning Increases HIF-1 Expression

Newborn rats exposed to hypoxia 1 day before ischemia are protected against ischemic brain injury.<sup>4–6</sup> In the present study, the brain protection afforded by hypoxic preconditioning was similar (~95%) to that of previous reports (not shown).<sup>4,5</sup> Because HIF-1 is specifically induced by hypoxia in vitro and in vivo,<sup>22,26,38,40</sup> we investigated the role of HIF-1 in

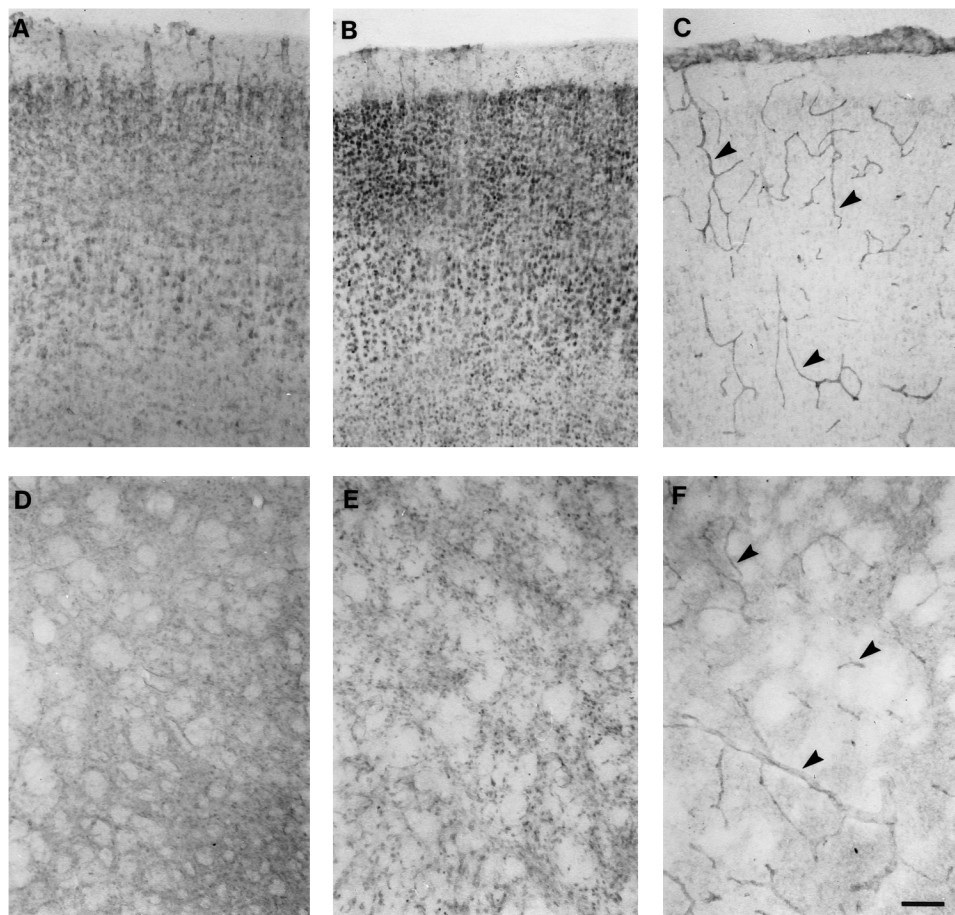


**Fig 3.** The effect of hypoxic preconditioning and hypoxia-ischemia on hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) protein expression in newborn rat brain. Coronal brain sections (50  $\mu$ m) were obtained from untreated newborn rats (A and B) or from animals subjected to hypoxic preconditioning (C and D; 8% O<sub>2</sub> for 3 hours) or hypoxic-ischemic injury (E and F; unilateral common carotid ligation combined with hypoxia). Immediately after hypoxic preconditioning, increased HIF-1 $\alpha$  immunostaining was observed throughout the brain, including the cerebral cortex (C) and the molecular layer of the CA1 through CA3 and dentate gyrus (DG) regions of the hippocampus (D) compared with that in untreated control animals (A and B). Despite a reduction of HIF-1 $\alpha$  protein expression in the brain parenchyma after hypoxia-ischemia, HIF-1 $\alpha$  immunostaining was still observed in the blood vessels throughout the cerebral cortex (E) and the hippocampus (F) ipsilateral to the carotid ligation. Scale bar = 200  $\mu$ m.

hypoxia-induced ischemic tolerance in brains from rat pups preconditioned by exposure to 8% O<sub>2</sub> for 3 hours. Because HIF-1 is degraded rapidly on reoxygenation,<sup>19,23,24,26</sup> brains were analyzed immediately after the end of hypoxia exposure and before reoxygenation. Northern blot analysis of brain tissue after preconditioning (see Fig 1, lane 2) showed a 1.7- to 2.3-fold increase in the HIF-1 $\alpha$  mRNA level, whereas the HIF-1 $\beta$  mRNA level was unchanged compared with that of untreated control brain tissue (see Fig 1, lane 1). Because induction of HIF-1 activity requires ongoing protein synthesis<sup>20</sup> and protein stabilization,<sup>19</sup> HIF-1 $\alpha$  and HIF-1 $\beta$  protein expression was evaluated by Western blot analysis (see Fig 2). As reported previously,<sup>26</sup> HIF-1 $\alpha$  and HIF-1 $\beta$  proteins were detected as a series

of isoforms with an apparent molecular mass of 113 to 121 kd or 91 to 94 kd, respectively. These isoforms may reflect different phosphorylation states, because HIF-1 $\alpha$  and HIF-1 $\beta$  (ARNT) protein sequences contain several phosphorylation sites. Indeed, HIF-1 activation and DNA binding activity require prior phosphorylation of the HIF-1 complex.<sup>41</sup> In the present study, hypoxia preconditioning produced a 25.8- to 62.9-fold increase in HIF-1 $\alpha$  protein levels and a 2.5- to 5.9-fold increase in HIF-1 $\beta$  protein levels (see Fig 2, lane 2). In animals subjected to hypoxia-ischemia, similar changes in both HIF-1 proteins were observed in the contralateral hemisphere, which was exposed to hypoxia only (without carotid ligation) (see Fig 2, lanes 3 and 5). Because HIF-1 $\alpha$  protein is specific to the





**Fig 4.** The effect of hypoxic preconditioning and hypoxia-ischemia on hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) protein expression in newborn rat cerebral cortex (A–C) and striatum (D–F). Photomicrographs of coronal brain sections (50  $\mu$ m) were obtained from untreated newborn rats (A and D) or from animals subjected to hypoxic preconditioning (B and E; 8% O<sub>2</sub> at 3 hours) or hypoxia-ischemic injury (C and F; unilateral common carotid ligation combined with hypoxia). Immediately after hypoxic preconditioning, HIF-1 $\alpha$  immunoreactivity was increased in the cerebral cortex (B) and the striatum (E) compared with that in untreated control regions (A and D). After hypoxia-ischemia, HIF-1 $\alpha$  immunostaining was observed in the blood vessels (arrows) despite an overall reduction of HIF-1 $\alpha$  expression in the brain parenchyma (C and F). Scale bar = 100  $\mu$ m.

HIF-1 heterodimer and changes in its expression closely parallel changes in HIF-1 DNA binding activity,<sup>22</sup> we further examined HIF-1 $\alpha$  protein expression by immunohistochemistry on brain sections. Consistent with our Western blot observations, hypoxia preconditioning produced an elevation of HIF-1 $\alpha$  immunostaining throughout the brain, including the cerebral cortex (see Figs 3C and 4B), hippocampus (see Fig 3D), striatum (see Fig 4E), thalamus, and hypothalamus (not shown). Moderate HIF-1 $\alpha$  protein expression was observed in blood vessels of hypoxic (preconditioned) animals (compare Fig 3B and D). Because of the substantial increase in HIF-1 $\alpha$  expression throughout the parenchyma after hypoxia, it was difficult to appreciate the degree of vascular expression in hypoxic brain. A similar pattern of immunostaining was observed in the contralateral hypoxic hemisphere of animals subjected to hypoxia-ischemia (not shown).

#### *Effect of Hypoxia-Ischemia on HIF-1 Expression*

We then investigated the effect of combined unilateral common carotid occlusion and systemic hypoxia in newborn rat brain. This model of perinatal hypoxia-ischemia in rat pups produces severe oxygen depletion<sup>42</sup> and subsequent brain injury in the hemisphere ipsilateral to the carotid occlusion.<sup>30,31</sup> Exposure to hypoxia alone (contralateral side) does not produce any evidence of cellular damage.<sup>4–6,30–32</sup> Northern blot analysis of brain extracts obtained from the ipsilateral (hypoxic-ischemic) hemisphere (see Fig 1, lane 3) showed a 1.4- to 2.5-fold increase in HIF-1 $\alpha$  mRNA, whereas the HIF-1 $\beta$  mRNA level remained unchanged compared with that of untreated control brain tissue (see Fig 1, lane 1). Western blot analysis also showed a modest 1- to 11.4-fold increase in HIF-1 $\alpha$  protein level and an unchanged or slightly decreased (0.2–0.34-fold) HIF-1 $\beta$  protein level after hypoxia-ischemia

(see Fig 2, lanes 4 and 6) compared with that of untreated control brain tissue (see Fig 2, lane 1). This induction of HIF-1 $\alpha$  protein expression observed after hypoxia-ischemia was always several times less than that produced by hypoxia alone in the contralateral hemisphere (see Fig 2, lanes 3 and 5) or by hypoxia preconditioning (see Fig 2, lane 2). Interestingly, immunohistochemistry showed that hypoxia-ischemia produced a decrease in parenchymal HIF-1 $\alpha$  immunostaining intensity, with a marked concurrent expression of HIF-1 $\alpha$  expression in the blood vessels throughout the ipsilateral brain regions, including the cerebral cortex (see Figs 3E and 4C), hippocampus (see Fig 3F), and striatum (see Fig 4F).

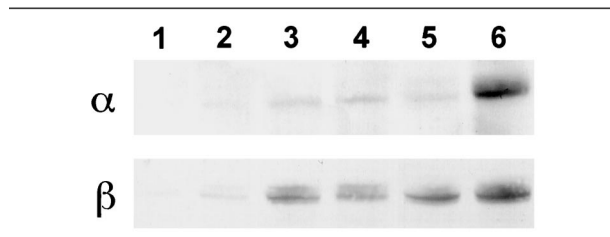
#### *DFX and CoCl<sub>2</sub> Pretreatment Induce HIF-1 Expression and Produce Ischemic Tolerance*

To further investigate the role of HIF-1 in ischemic tolerance, we studied the effect of known HIF-1 inducers other than hypoxia. In vitro studies have shown that HIF-1 is strongly induced by DFX or CoCl<sub>2</sub>.<sup>25–27</sup> Therefore, we examined HIF-1 expression in brains from rat pups administered either DFX or CoCl<sub>2</sub>. At the doses used, there was no overt adverse physiological or behavioral change in newborn rats administered either DFX or CoCl<sub>2</sub> compared with vehicle-injected animals. In particular, animals receiving DFX or CoCl<sub>2</sub> demonstrated intact grasping and righting reflexes, normal ambulation, respiration, suckling, and tail pinch response. Brains from these animals showed no abnormalities on Nissl staining, which is consistent with previous reports.<sup>43,44</sup> Western blot analysis showed that DFX treatment produced a small 1.3- to 2.4-fold increase in HIF-1 $\alpha$  protein level (Fig 5, lanes 3 and 4), whereas HIF-1 $\beta$  levels were elevated 0.7- to 2.1-fold in 7 of 13 animals treated with DFX, irrespective of the exposure time. In contrast, CoCl<sub>2</sub> administration in-

creased HIF-1 $\alpha$  (0.5–4.3-fold) and HIF-1 $\beta$  (9.4–10.0-fold) proteins 1 hour after injection (see Fig 5, lane 5) compared with those in time-matched vehicle-injected controls (see Fig 5, lane 1). At 3 hours after injection, peak levels of HIF-1 $\alpha$  (4.3–38.7-fold) and HIF-1 $\beta$  (9.7–17.4-fold) proteins were observed (see Fig 5, lane 6) compared with those in the corresponding controls (see Fig 5, lane 2). Six hours after CoCl<sub>2</sub> injection, HIF-1 $\alpha$  and HIF-1 $\beta$  protein levels showed a 38% decline compared with the levels found at the 3-hour time point (not shown).

To evaluate the relation between the level of HIF-1 expression and tolerance against ischemic brain damage, we investigated whether DFX and CoCl<sub>2</sub> pretreatment (which induce HIF-1 expression) reduced hypoxic-ischemic brain injury in newborn rats. As mentioned earlier, hypoxia-ischemia in the P7 newborn rat is characterized by injury to the hemisphere ipsilateral to the carotid ligation. Damage ranges from loss of a few isolated cells to gross infarction in the cortex, hippocampus, striatum, thalamus, and white matter ipsilateral to the ligation.<sup>30,31</sup> As shown for the control animals (vehicle) in Figure 6, moderate to severe injury is more common than little or no damage.<sup>30,31</sup> Indeed, significant infarction, manifested as  $32.3 \pm 4.0\%$  (vehicle group, no DFX; see Fig 6, left panel) and  $43.0 \pm 3.7\%$  (vehicle group, no CoCl<sub>2</sub>; see Fig 6, right panel) reductions in hemispheric weight ipsilateral to the carotid ligation, was noted in vehicle-treated animals 1 week after hypoxia-ischemia. These findings are consistent with those of previous reports<sup>4–6,31</sup> and are in agreement with the Nissl staining observations (not shown). Preconditioning with DFX or CoCl<sub>2</sub> resulted in a  $14.1 \pm 3.0\%$  and  $10.6 \pm 3.4\%$  loss in ipsilateral hemispheric weights, which represents 56% (DFX) and 75% (CoCl<sub>2</sub>) protection compared with that in vehicle-injected littermate controls (see Fig 6).

*Fig 5. Western blot analysis of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) and HIF-1 $\beta$  proteins in the brain of newborn rats administered a single intraperitoneal injection of desferrioxamine (DFX, 200 mg/kg; lanes 3 and 4), cobalt chloride (CoCl<sub>2</sub>, 60 mg/kg; lanes 5 and 6), or vehicle (lanes 1 and 2). Brains were analyzed either 1 hour (lanes 1, 3, and 5) or 3 hours (lanes 2, 4, and 6) after injection. Equal amounts of protein samples (150  $\mu$ g) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting with rabbit anti-HIF-1 $\alpha$  and anti-HIF-1 $\beta$  polyclonal antibodies.*



#### **Discussion**

Newborn rats subjected to hypoxia preconditioning (8% O<sub>2</sub> for 3 hours) sustain markedly reduced brain damage after cerebral hypoxia-ischemia compared with animals not exposed to prior hypoxia.<sup>4–6</sup> Such preconditioning produces moderate tissue hypoxia,<sup>42</sup> which has relatively few effects on regional cerebral blood flow,<sup>45</sup> water content,<sup>46</sup> calcium uptake,<sup>47</sup> protein synthesis,<sup>48</sup> and cellular integrity.<sup>4,31</sup> The present study provides evidence that HIF-1 induction may be an important component of an adaptive response elicited by hypoxia preconditioning in neonatal rat brain. Immediately after hypoxic preconditioning, levels of HIF-1 $\alpha$  mRNA and of HIF-1 $\alpha$  and HIF-1 $\beta$  proteins were markedly increased throughout the newborn rat brain, including the cerebral cortex, hippocampus, striatum, and thalamus, which are the regions known to be most susceptible to neonatal hypoxic-ischemic brain injury.

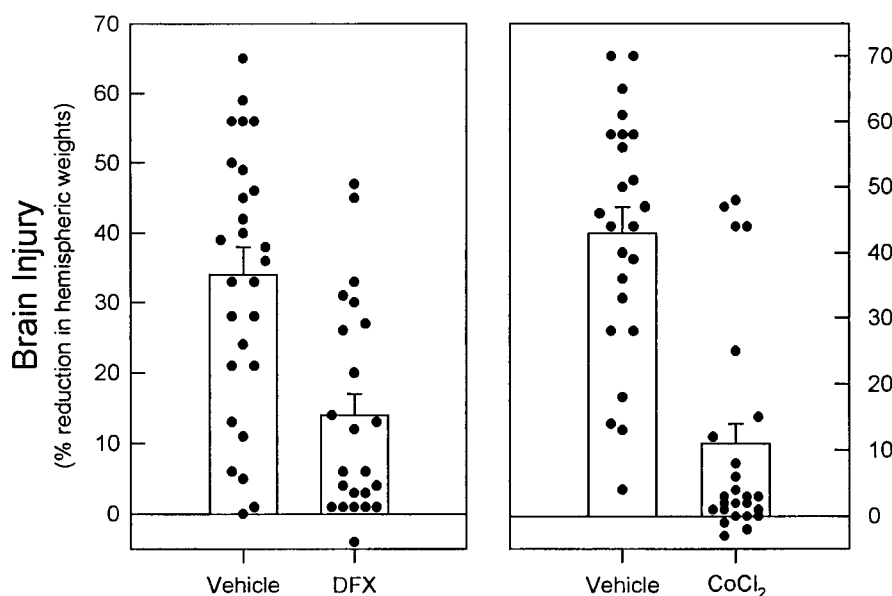


Fig 6. Pretreatment with desferrioxamine (DFX) or cobalt chloride (CoCl<sub>2</sub>) confers protection from hypoxic-ischemic brain injury in newborn rat brain. Rats at postnatal day (P) 6 either received a single intraperitoneal injection of DFX (left panel) or CoCl<sub>2</sub> (right panel), although control littermates received vehicle (both panels). Twenty-four hours after injection, rat pups were subjected to hypoxia-ischemia. Animals were then allowed to recover for 1 week before analysis (at P14). Brain injury in CoCl<sub>2</sub>- and DFX-treated animals was significantly less ( $p < 0.005$ ) than that measured in control littermates injected with vehicle and that were not preconditioned. Each data point represents one animal. Mean and SD for each group are represented as histograms overlapping each distribution. Brain injury was quantified as the percentage of reduction in hemispheric weight in the ipsilateral hemisphere. Significant differences between a treatment group and the vehicle-treated group were tested using the two-tailed Mann-Whitney nonparametric test. For DFX (14.1%) versus vehicle (32.3%), the probability value was less than 0.005. For CoCl<sub>2</sub> (10.6%) versus vehicle (43.0%), the probability value was less than 0.0001.

ry.<sup>30,31</sup> In addition, preconditioning with either CoCl<sub>2</sub> or DFX induced HIF-1 expression and resulted in neuroprotection.

In the present study, constitutive (normoxic) levels of HIF-1 $\alpha$  and HIF-1 $\beta$  mRNA and protein were detected in healthy newborn rat brain, which is consistent with the constitutive HIF-1 mRNA expression and HIF-1 DNA binding activity recently observed in primary neuronal cultures.<sup>9,27,49</sup> In addition, *in vivo* studies have shown constitutive expression of HIF-1 subunits in rat and mouse brain and in different human and rodent organs.<sup>17,36–40,50</sup> This constitutive *in vivo* HIF-1 expression in brain and other tissues is likely to be due to the moderate hypoxic state (estimated mean PO<sub>2</sub> value of 2–5%) found under normoxic conditions in most mammalian tissues, including the cerebral cortex.<sup>22,51</sup> Because the HIF-1 response *in vitro* has been shown to increase exponentially as the oxygen concentration declines below 6%,<sup>22</sup> a small reduction in the oxygen level in the brain or other organs would be expected to induce HIF-1 expression even more. This is consistent with activation of HIF-1-responsive reporter plasmids<sup>49</sup> and increased HIF-1 DNA binding activity<sup>9</sup> in hypoxic neuronal cultures. Accordingly, increased expression of HIF-1 subunits

has been shown in the brain, lung, kidney, heart, liver, and spleen from mice and rats exposed to moderate systemic hypoxia.<sup>37,38,40</sup> In the present study, the marked upregulation of HIF-1 protein subunits in newborn rat brain by systemic hypoxia is consistent with the hypothesis that hypoxia is the primary stimulus for HIF-1 induction in all mammalian tissues.<sup>10,37,38,40</sup>

In newborn rat brain, the expression of several genes such as the immediate early genes *fos* and *jun*<sup>52</sup> and the stress proteins HSP72<sup>52,53</sup> and HSP32<sup>30</sup> is unaffected by hypoxia treatment, whereas combined hypoxia-ischemia results in a marked upregulation. In the present study, HIF-1 does not follow the classic definition of a stress gene, because HIF-1 $\alpha$  protein is induced by hypoxic preconditioning and repressed by hypoxia-ischemia despite a small elevation of HIF-1 $\alpha$  mRNA. Hypoxia-ischemia in newborn rat brain produces severe oxygen depletion<sup>42</sup> and decreased energy metabolites,<sup>54</sup> which lead to decreased overall protein synthesis in the ischemic brain parenchyma.<sup>48</sup> Because HIF-1 activation requires *de novo* protein synthesis,<sup>20,21</sup> an impairment of protein synthesis during hypoxia-ischemia<sup>48</sup> may account for the lack of induction of HIF-1 protein expression in the ischemic newborn brain. These observations are in agreement with



recent findings in adult rat brain showing downregulation of HIF-1 $\alpha$  mRNA and protein expression in infarcted regions after permanent cerebral focal ischemia.<sup>37</sup> Interestingly, expression of HIF-1 $\alpha$  protein was found in the microvasculature of the ischemic core region. Because blood vessels are usually more resistant to ischemic brain damage, they are still capable of synthesizing proteins that may be involved in their survival such as HSP72<sup>53</sup> and HSP32.<sup>30</sup> The expression of HIF-1 in blood vessels after ischemia supports its proposed role in angiogenesis and glucose transport through upregulation of VEGF<sup>36,50,55–58</sup> and the glucose transporters.<sup>11,59</sup>

To begin to examine the possible protective role of HIF-1 in hypoxia-induced tolerance in newborn rat brain, we tested whether DFX and CoCl<sub>2</sub>, which are two known inducers of HIF-1 expression and activity in vitro,<sup>25–27,49</sup> also induced HIF-1 in rat brain. We also wanted to correlate the induction of HIF-1 with its potential protective effect. As predicted by the in vitro data, administration of either DFX or CoCl<sub>2</sub> increased the level of HIF-1 protein subunits in newborn rat brain. Interestingly, CoCl<sub>2</sub> was a much better inducer of HIF-1 expression than DFX. Whereas CoCl<sub>2</sub> treatment increased both HIF-1 $\alpha$  and HIF-1 $\beta$  protein levels, DFX treatment resulted in HIF-1 $\beta$  protein levels several times greater than the modest increase in HIF-1 $\alpha$  protein level. Because HIF-1 $\alpha$  is the limiting factor that determines the level of HIF-1 DNA binding and transcriptional activity within cells,<sup>22</sup> the small induction of HIF-1 $\alpha$  protein expression by DFX despite increased expression of HIF-1 $\beta$  may account for the more modest neuroprotection afforded by DFX preconditioning compared with CoCl<sub>2</sub> or hypoxia treatments. The possibility that mechanisms other than HIF-1 induction (eg, decreased brain iron and free radicals, induction of stress proteins such as HSP32 by CoCl<sub>2</sub>) may be involved in this protection cannot be excluded. Although the levels of DFX or CoCl<sub>2</sub> in neonate brain were not measured in the present study, previous observations suggest that the differences in the ability of DFX and CoCl<sub>2</sub> to stimulate HIF-1 expression may be the result of differences in their ability to cross the blood–brain barrier. Whereas cobaltous ions seem to readily cross the blood–brain barrier in adult rats and human beings,<sup>44,60</sup> it is not clear how well DFX might cross the barrier. In one study,<sup>43</sup> the brain concentration of DFX was found to closely parallel the serum level in the newborn rat after a single subcutaneous injection of DFX. Conversely, another study found DFX immunoreactivity only in the choroid plexus and ependymal cells 24 to 72 hours after intraperitoneal injections of DFX in P0, P5, P11, and adult mice.<sup>61</sup> Consistent with this observation, although the serum half-life of DFX has been estimated at

3 hours,<sup>43</sup> our results show only a modest HIF-1 $\alpha$  level at all time points studied.

Cells respond to hypoxia by modulating the expression of adaptive or pathological genes depending on the severity and duration of the stimulus.<sup>10,56</sup> HIF-1 is an important component of the adaptive response to hypoxia.<sup>10,36</sup> It is involved in the modulation of developmental cellular processes and the regulation of cellular oxygen homeostasis.<sup>10,36,56</sup> More recently, the role of HIF-1 in maladaptive processes has also been suggested. In vitro studies have shown that exposure to hypoxia or hypoxia-mimetics resulted in HIF-1 $\alpha$  association with and stabilization of p53,<sup>62</sup> leading to inhibition of HIF-1 transactivating activity.<sup>63</sup> Moreover, the disruption of HIF-1 signaling attenuated hypoxia-induced apoptotic cell death in embryonic stem cells or cortical neurons.<sup>49,58</sup> Interestingly, apoptosis induced by glutathione depletion in cultured neurons was prevented by stimulation of HIF-1 activity and HIF-1 target gene expression with hypoxia-mimetics.<sup>27</sup> These differences in HIF-1-mediated effects may depend on the type of cell used as well as the type and severity of death stimulus. In the present study, it seems unlikely that HIF-1 induction would promote apoptosis after hypoxic preconditioning, because this treatment does not cause any brain damage and no delayed cell death.<sup>4–6,30–32</sup> Although the expression of HIF-1 was generally repressed immediately after hypoxia-ischemia, it is possible that the basal HIF-1 expression remaining in regions just outside the ischemic core could be involved in the remote delayed cell death observed after neonatal hypoxia-ischemia.<sup>32</sup>

Targeted disruption of HIF-1 $\alpha$ <sup>36,55,58,64</sup> or HIF-1 $\beta$ <sup>65,66</sup> impairs the response of HIF-1 and its target genes to hypoxia or DFX and produces embryonic lethality associated with profound neurological and cardiovascular abnormalities. In the present study, preconditioning newborn rats with hypoxia or hypoxia-mimetics (DFX and CoCl<sub>2</sub>) produced a long-lasting adaptive and protective response that is likely to involve increased HIF-1 target genes as a result of HIF-1 activation. Indeed, a recent study showed that DFX or CoCl<sub>2</sub> exposure protected neuronal cultures against glutathione depletion-induced cell death.<sup>27</sup> Interestingly, this neuroprotection was associated with enhanced HIF-1 DNA binding activity and increased expression of HIF-1 $\alpha$ , HIF-1 $\beta$ , and HIF-1 target genes, including erythropoietin and some glycolytic enzymes.<sup>27</sup> Similarly, DFX or CoCl<sub>2</sub> application in different mammalian cell lines also induced the expression of several HIF-1 target genes like erythropoietin,<sup>25</sup> glucose transporter-1,<sup>11,67</sup> VEGF,<sup>58,67</sup> and the glycolytic enzymes.<sup>10,68</sup> In vivo, increased glucose transporter-1 mRNA has been reported in cerebral vessels after hypoxia and hypoxia-ischemia in newborn rat brain,<sup>59</sup> which is consistent with our findings of increased HIF-1 $\alpha$  in blood vessels after the

same treatments. Furthermore, chronic  $\text{CoCl}_2$  administration in drinking water increases the expression of glucose transporter-1 and VEGF throughout rat brain.<sup>44</sup> Increased VEGF expression has also been observed in ischemic mouse retina.<sup>50</sup> After permanent focal ischemia in adult rats, expression of HIF-1 $\alpha$ , the glycolytic enzymes, and glucose transporter-1 increased significantly in the peri-infarct "hypoxic" penumbra.<sup>37</sup> Similarly, upregulation of erythropoietin<sup>69</sup> and VEGF<sup>57,70,71</sup> has been reported in the peri-infarct penumbra after focal ischemia in mice, rats, and human beings. Recent data support a direct protective role of some HIF-1 target genes against cerebral ischemia. Topical application of VEGF on the surface of perfused rat brain reduces infarct size after ischemia,<sup>72</sup> and pretreatment with erythropoietin protects against glutamate-induced death in neuronal cultures<sup>73</sup> and learning disability and neuronal loss in the hippocampal CA1 region induced by global ischemia in gerbils.<sup>74</sup> It is possible that delayed expression of glucose transporters and glycolytic enzymes after hypoxic preconditioning helps to prevent the secondary energy depletion reported during the early recovery phase after neonatal hypoxia-ischemia.<sup>6</sup> Further studies should elucidate whether the increased HIF-1 expression observed after hypoxia,  $\text{CoCl}_2$ , or DFX preconditioning is associated with increased HIF-1 target gene expression.

In summary, this study showed that hypoxia and hypoxia-mimetics ( $\text{CoCl}_2$  and DFX) induced HIF-1 expression in newborn rat brain. In addition, we demonstrated that the level of HIF-1 $\alpha$  expression correlated with the degree of brain protection afforded after each preconditioning treatment (hypoxia >  $\text{CoCl}_2$  > DFX). Thus, increased expression of HIF-1 by hypoxia preconditioning could induce several defense mechanisms such as increased glucose transport, glycolysis, and vascular growth, all of which may contribute to protecting the brain during ischemia.

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