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Neuroprotection by Hypoxic Preconditioning Involves Upregulation of Hypoxia-Inducible Factor-1 in a Prenatal Model of Acute Hypoxia

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The molecular pathways underlying the neuroprotective effects of preconditioning are promising, potentially drugable targets to promote cell survival. However, these pathways are complex and are not yet fully understood. In this study we have established a paradigm of hypoxic preconditioning based on a chick embryo model of normobaric acute hypoxia previously developed by our group. With this model, we analyzed the role of hypoxia-inducible factor-1α (HIF-1α) stabilization during preconditioning in HIF-1 signaling after the hypoxic injury and in the development of a neuroprotective effect against the insult. To this end, we used a pharmacological approach, based on the in vivo administration of positive (Fe^{2+}, ascorbate) and negative ($CoCl_2$) modulators of the activity of HIF-prolyl hydroxylases (PHDs), the main regulators of HIF-1. We have found that preconditioning has a reinforcing effect on HIF-1 accumulation during the subsequent hypoxic injury. In addition, we have also demonstrated that HIF-1 induction during hypoxic preconditioning is necessary to obtain an enhancement in HIF-1 accumulation and to develop a tolerance against a subsequent hypoxic injury. We provide in vivo evidence that administration of Fe^{2+} and ascorbate modulates HIF accumulation, suggesting that PHDs might be targets for neuroprotection in the CNS.

Key words: hypoxia; preconditioning; prolyl hydroxylases; developing brain

In the context of cerebral hypoxia/ischemia, several methods of preconditioning, including heat shock, oxidative stress, hypoxia, and ischemia itself, have been investigated (Gidday, 2006). Nevertheless, the complex molecular mechanisms through which preconditioning causes tolerance have only begun to be unravelled. This might be attributable to the remarkable heterogeneity found in the literature regarding both the stimulus used for preconditioning and the model of injury. In particular, the intricate conditions present in ischemic insults, i.e., hypoxia, hypoglycemia, hypercapnia, and inflammation (Lipton, 1999), create an entangled context for the elucidation of the molecular pathways underlying the neuroprotective effects. Therefore, in this study, we established a paradigm of hypoxic preconditioning based on a simplified system of injury: a chick embryo model of normobaric acute hypoxia developed by our group (Rodriguez Gil et al., 2000). In this model, CO_2 and glucose levels are normal, no invasive procedures to set hypoxia are required, and maternal effects are absent. With this experimental model, we have previously determined that hypoxia induces an activation of the intrinsic apoptotic pathway in neurons (Pozo Devoto et al., 2008), preceded by nitric oxide (NO)-dependent mitochondrial damage (Giusti et al., 2008) and the activation of JNK and p38 MAP kinases (Vacotto et al., 2008).

Until now, several key molecules and signaling pathways have been connected to preconditioning in the nervous system, including MAPKs (Nishimura et al., 2003) PI3/Akt (Ruscher et al., 2002), and hypoxia-inducible factor-1 (HIF-1; Sharp et al., 2004; LaManna, 2007). The latter has attracted particular interest because it plays a central role in oxygen homeostasis.

The transcription factor HIF-1 is a heterodimer composed of an O_2-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit (Semenza, 2007). In well-oxygenated cells, HIF-1α is an exceptionally short-lived protein that is continuously synthesized, polyubiquitinated, and targeted for degradation

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(Maxwell et al., 1999; Ohh et al., 2000). HIF-specific prolyl hydroxylases (here referred to as *PHDs*) catalyze the O_2-dependent prolyl hydroxylation reaction and are consequently critical regulators of HIF-1 (Epstein et al., 2001).

Years after the use of $CoCl_2$ to induce HIF-1 under normoxic conditions, it was shown that this effect relies on the inhibition of PHDs (Yuan et al., 2003). Divalent metallic ions, such as Co^{2+} and Ni^{2+}, substitute for the ferrous iron located in the hydrophobic active site that is required for the assembly of the enzyme into its active conformation (McDonough et al., 2006).

In the hydroxylation reaction, one oxygen atom is added to a peptidyl proline to form hydroxyproline, whereas the other is used in a coupled decarboxylation reaction that converts 2-oxoglutarate to succinate (Hirsila et al., 2003). During this process, Fe^{2+} is transiently oxidized to Fe^{4+} and then restored to the Fe^{2+} state. However, 2-oxoglutarate can also be converted into succinate in an uncoupled manner, i.e., without hydroxylation of a peptide substrate. In this context, Fe^{2+} is oxidized to Fe^{3+} (Counts et al., 1978), and ascorbate is required to reduce it back to Fe^{2+} for the enzyme to be recycled (Nietfeld and Kemp, 1981). In accordance with this, it has been shown that intracellular ascorbate and Fe^{2+} levels can also affect PHD enzymatic activity and HIF-1α protein accumulation (Knowles et al., 2003).

In addition to the establishment of a model of prenatal hypoxic preconditioning, the present study sought to analyze the role of HIF-1α stabilization during the preconditioning phase in both HIF-1 signaling after the hypoxic injury and development of a neuroprotective effect against the insult. To this end, we used a pharmacological approach, based on the in vivo administration of positive (Fe^{2+}, ascorbate) and negative ($CoCl_2$) regulators of PHDs activity.

MATERIALS AND METHODS

Animals and Hypoxic Treatments

White leghorn fertile chicken (*Gallus gallus domesticus*) eggs were obtained from a local hatchery and incubated at 38°C and 60% relative humidity. Global hypoxic treatments were preformed as described previously, with minor modifications (Rodriguez Gil et al., 2000). Briefly, eggs were vertically placed in a 10-liter plastic chamber inside the incubator and subjected to a stream of 8% O_2/92% N_2 over 40 min (hypoxic preconditioning) or 60 min (injury) at a flow rate of 1 liter/min. The chamber contained retention valves to allow escape of gases in excess while avoiding mixing with atmospheric air and a storage space with calcium hydroxide to absorb CO_2 formed during the treatment. After that, eggs were returned to normoxic conditions in the incubator, and allowed to recover for different lengths of time.

To allow the in vivo administration of different pharmacological agents, some eggs were windowed following a modification of the Hamburger procedure (Hamburger, 1960) 1 day before the hypoxic treatment. In brief, a rectangular area of the shell roughly 4–6 mm^2 in size was removed, and the window was covered with Micropore tape (3M). When appropriate, the tapes were removed, and 100 μl of a pharmacologic agent solution or vehicle (saline) was applied to the vascularized chorioallantoic membrane. Final concentrations were calculated considering a final volume of 50 ml/egg.

Experimental Design

The experimental design includes three different but related procedures, schematically depicted in Figure 1. On embryonic day (ED) 11, embryos were subjected to hypoxic preconditioning, followed by a hypoxic injury 24 hr later, on ED 12 (Fig. 1A). In another group, hypoxic preconditioning was substituted by $CoCl_2$ (Sigma, St. Louis, MO) administration on ED 11 (Fig. 1B). Three final concentrations of $CoCl_2$ were used: $1 \cdot 10^{-3}$ mM, $1 \cdot 10^{-2}$ mM, and $1 \cdot 10^{-1}$ mM. In some other cases, 20 min before hypoxic treatment, embryos received a dose of Fe^{2+}, in the form of $FeCl_2$ solution (Mallinckrodt, Hazelwood, MO) or ascorbate (Carlo Erba; Fig. 1C). The final concentrations used for Fe^{2+} were $5 \cdot 10^{-4}$ mM, $5 \cdot 10^{-3}$ mM, and $5 \cdot 10^{-2}$ mM, whereas the final concentrations used for ascorbate were $5 \cdot 10^{-3}$ mM, $5 \cdot 10^{-2}$ mM, and $5 \cdot 10^{-1}$ mM.

Other groups, not depicted in Figure 1, were used for control purposes and are specified in Results. They include embryos that were not preconditioned and subjected to the hypoxic injury and, conversely, embryos preconditioned and not subjected to the hypoxic insult.

Tissue Preparation

In all cases, the optic lobes were used. They are bilateral mesencephalic structures involved in visual processing and exquisitely developed in avian species. Chick embryos were sacrificed by decapitation, and optic lobes were quickly removed and placed in ice-cold PBS.

Purified nuclei and pure soluble nuclear fractions derived from them were prepared by an improved method recently developed by our group and described elsewhere (Giusti et al., 2009). Optic lobes of six embryos were pooled to obtain a single sample.

Cytosolic fractions were obtained by homogenizing optic lobes from four embryos in ice-cold buffer containing 320 mM sucrose, 20 mM Tris-HCl (pH 7.4), 1 mM ethylenediaminetetracetic acid, 1 mM ethyleneglycoltetracetic acid, 50 mM NaF, 1 mM Na_3VO_4, 1 mM amonium molybdate, 10 μg/ml each of proteases inhibitors (aprotinin, leupeptin, pepstatin), and 1 mM phenylmethanesulfonyl fluoride. Homogenates were pelleted at 100,000*g* for 30 min, and the supernatant was collected as cytosolic fractions and stored at −20°C. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

For DNA fragmentation assays, chicken embryos were decapitated, and the brains rapidly removed and fixed for 12 hr in 4% paraformaldehide in PBS. After rinsing with PBS, brains were dehydrated with a graded series of ethanol, then xylene and xylene/paraffin, and then embedded in paraffin. Then, brains were cut into 5-μm-thick sections. The optic

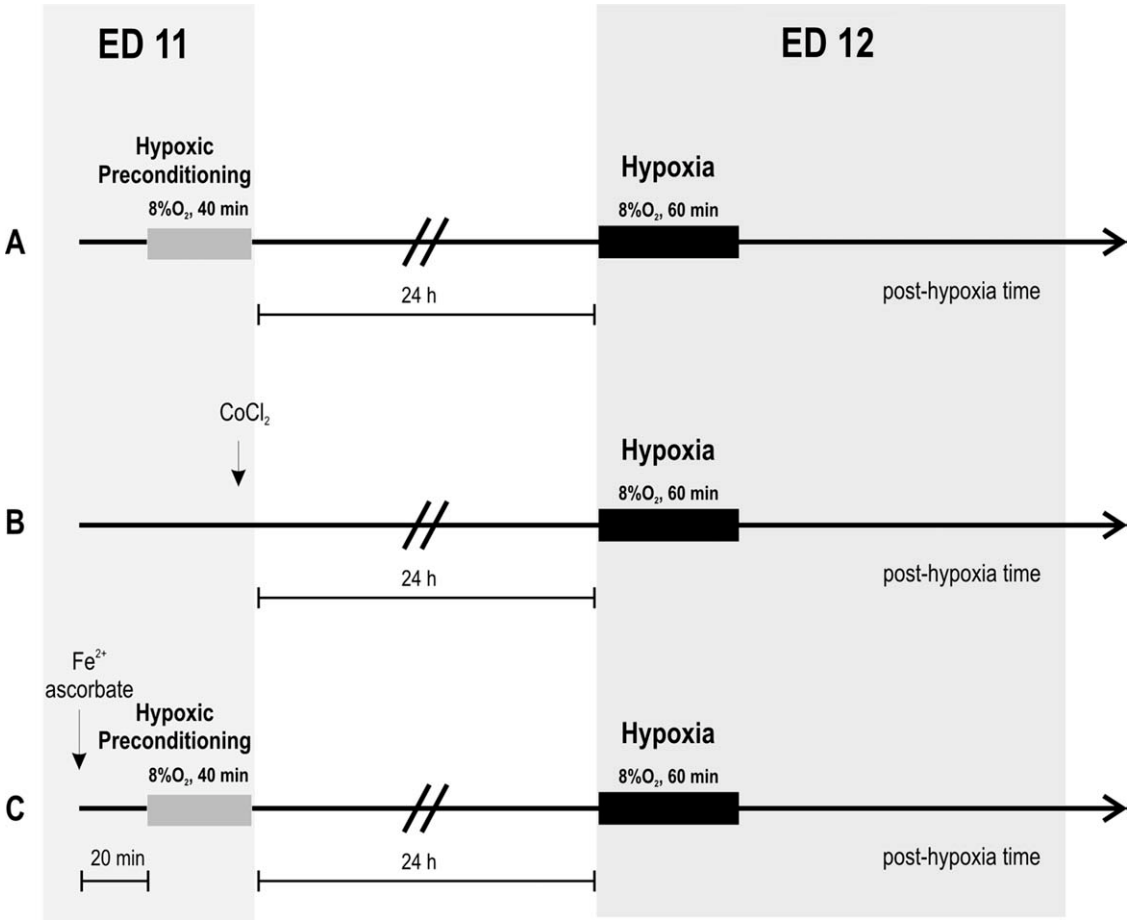

Fig. 1. Experimental design. **A:** Hypoxic preconditioning in an acute hypoxic injury model. Preconditioning was performed on embryonic day (ED) 11, and hypoxic injury was performed 24 hr later, on ED 12. **B:** In some cases, hypoxic preconditioning was replaced by preconditioning with $CoCl_2$. **C:** In some cases, Fe^{2+} or ascorbate was administered 20 min before hypoxic preconditioning.

lobes were placed such that the sections were parallel to the optic lobe longitudinal anatomical axis.

Western Blotting

Soluble nuclear fractions and cytosolic were denatured in loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% β-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue) at 100°C for 10 min and then separated by electrophoresis on a SDS-polyacrylamide gel (Bio-Rad, Richmond, CA) at 10% for HIF-1α and at for 18% cytochrome c. Proteins were then transferred to a PVDF membrane (Millipore, Bedford, MA). After blocking in 4% milk in phosphate-buffered saline with Tween 20 (T 0.05% in 1× PBS) for 1 hr, membranes were incubated with the primary antibody overnight at 4°C followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Chemicon, Temecula, CA). For primary antibodies, the dilutions used were 1:1,000 for anti-HIF-1α, anticytochrome c, antihistone H3, and antiactin monoclonal antibodies (Chemicon). When relevant, purified horse cytochrome c (Sigma-Aldrich, St. Louis, MO) was used as a positive control.

Bands were detected by chemiluminescence using ECL Kit (Amersham Pharmacia Biotech). Optical density of the radioautograms was quantified with Gel-Pro Analyzer 3.1 (Media Cybernetics, Bethesda, MD).

Immunogold Labeling and Electron Microscopy

Purified nuclei were fixed in cold 4% paraformaldhyde w/v and 0.5% w/v glutaraldehyde dissolved in 0.1 M phosphate buffer (pH 7.2–7.4) for 2 hr at 4°C. Each sample was then postfixed in the same buffer solution supplemented with 1% w/v osmium tetroxide. After two 15-min washes with distilled water, samples were immersed for 2 hr in 5% (w/v) uranyl acetate and immediately dehydrated and embedded in LR-White resin (London Resin Co., Hampshire, United Kingdom). Ultrathin sections (70–90 nm) were mounted on nickel grids, and nonspecific binding sites were blocked by incubating the sections for 45 min on a drop of 0.2% BSA and 0.1% Tween 20 in PBS at RT. After a short rinse in PBS, grids were incubated onto a drop of primary mouse anti-HIF-1α (diluted 1/20) in a moist chamber at 4°C over-

night. After three short rinses in PBS, sections were incubated with secondary 10-nm gold-coupled goat anti-mouse antibody (Sigma-Aldrich) diluted 1/50 for 1 hr. The sections were then stained with uranyl acetate and lead citrate (Reynolds, 1963). Samples were observed and photographed with a C10 Zeiss electron microscope. Quantification of immunogold labeling was performed by a blinded investigator. The negative control, omitting the primary antibody, produced no labeling.

DNA Fragmentation Assay

Detection of DNA double-strand breaks by TUNEL was performed according to the instructions of the manufacturer (Promega, Madison, WI). Sections were deparaffinized and rehydrated. The sections were then incubated in equilibrium buffer for 10 min, followed by reaction mix (equilibrium buffer, nucleotide mix with fluorescein conjugated dUTPs and terminal deoxynucleotidyl transferase) for 60 min. The reaction was stopped with 2× SSC for 15 min, and the sections were washed with PBS-Tween 20, counterstained with Hoechst 33258 for 10 min, and finally mounted with fluorescent mounting medium. On ED 12, the OL longitudinal anatomical axis runs parallel to the developmental gradient axis, so sections have a rostral pole and a caudal pole; the rostral occurs earlier in development than the caudal. To eliminate any developmental variables, all measurements were made halfway between the rostral and the caudal poles. Images were taken for each treatment with an Olympus BX50 microscope (Olympus, Tokyo, Japan) and were acquired with a CCD camera . A region of interest was selected for each image, such that the optic tectum from pia to ventricle was covered completely. Positive cell counting was performed and expressed as cells per square millimeter. Eight sections were analyzed and averaged for each embryo. All the quantification and morphological analyses were performed in ImageJ software (National Institutes of Health).

RESULTS

Hypoxic Preconditioning Has Neuroprotective Effects

As a first step, we determined whether an acute hypoxic exposure of 40 min, 24 hr before the hypoxic treatment (hypoxic preconditioning), had neuroprotective effects in our experimental model (Fig. 1A). The putative protective effect of hypoxic preconditioning was evaluated by the analysis of two markers of apoptotic cell death fully characterized in our experimental model of injury: cytochrome c release determined by Western blot of cytosolic fractions and DNA fragmentation assessed by TUNEL staining of histological sections. The effects of hypoxic preconditioning on those markers were tested at 4 and 6 hr posthypoxia, when cytochrome c release and TUNEL positive nuclei, reached their maximum values, respectively (Pozo Devoto et al., 2008). We observed that hypoxic preconditioning was able to reduce the intensity of both parameters significantly (Fig. 2), demonstrating that this treatment is neuroprotective against a hypoxic injury.

HIF-1 Induction After Hypoxic Preconditioning.

Next, we aimed to determine the molecular pathways involved in the neuroprotection afforded by the hypoxic preconditioning. Given the central role of HIF-1 in oxygen homeostasis, we hypothesized that this transcription factor could be a key mediator of the observed neuroprotective effect. To test this, the kinetics of HIF-1α accumulation after hypoxia were assessed by Western blot in pure soluble nuclear fractions. As expected, a rapid and strong accumulation of HIF-1α was observed at the end of the hypoxia (Fig. 3A). This accumulation was transient and could not be detected after the first 30 min of reoxygenation. The significant increase in HIF-1α content at 0 hr posthypoxia was further confirmed with gold immunolabeling of purified nuclei (Fig. 3B). Of particular importance is the fact that a significant increase in HIF-1α was also detected after a preconditioning-like hypoxic treatment (Fig. 3C), raising the possibility that the effects of preconditioning could be initially signaled in an HIF-dependent manner.

Effect of Hypoxic Preconditioning on HIF-1 Response After Hypoxia

Interestingly, we found that hypoxic preconditioning had an effect on the activation of HIF-1 signaling after the hypoxic injury. When the intensity of HIF-1 induction was compared between preconditioned and nonpreconditioned embryos, we observed that preconditioning resulted in an enhancement of ~30% after the hypoxic treatment (Fig. 4).

To determine whether there is a causal relationship between HIF-1 activation during hypoxic preconditioning and the enhancement of this activation after hypoxia, the following treatments were performed. First, hypoxic preconditioning was replaced by $CoCl_2$ administration (preconditioning with $CoCl_2$; Fig. 1B). This compound induces HIF-1 through the inactivation of PDHs even under normoxic conditions (Wang et al., 1995). Second, hypoxic preconditioning was preceded by the administration (20 min before) of ascorbate or Fe^{2+} (Fig. 1C). Each of these compounds diminishes HIF accumulation by stimulating the activity of PDHs (Knowles et al., 2003, 2006). Stabilization of HIF-1α by preconditioning with $CoCl_2$ and the diminishment of its accumulation resulting from Fe^{2+} or ascorbate treatment were checked by Western blot (data not shown). These treatments allow the discrimination of HIF-dependent effects from others also present during the hypoxic preconditioning.

We observed that preconditioning with $CoCl_2$ reproduces the enhancement of HIF-1 activation induced by the hypoxic preconditioning in a dose-dependent manner (Fig. 5A). In addition, we found that the negative modulators of HIF-1, ascorbate or Fe^{2+}, reverse this enhancement also in a dose-dependent manner (Fig. 5B). These results suggest that HIF-1 activation during preconditioning is necessary for the enhancement of this activation after hypoxic treatment on the following day.

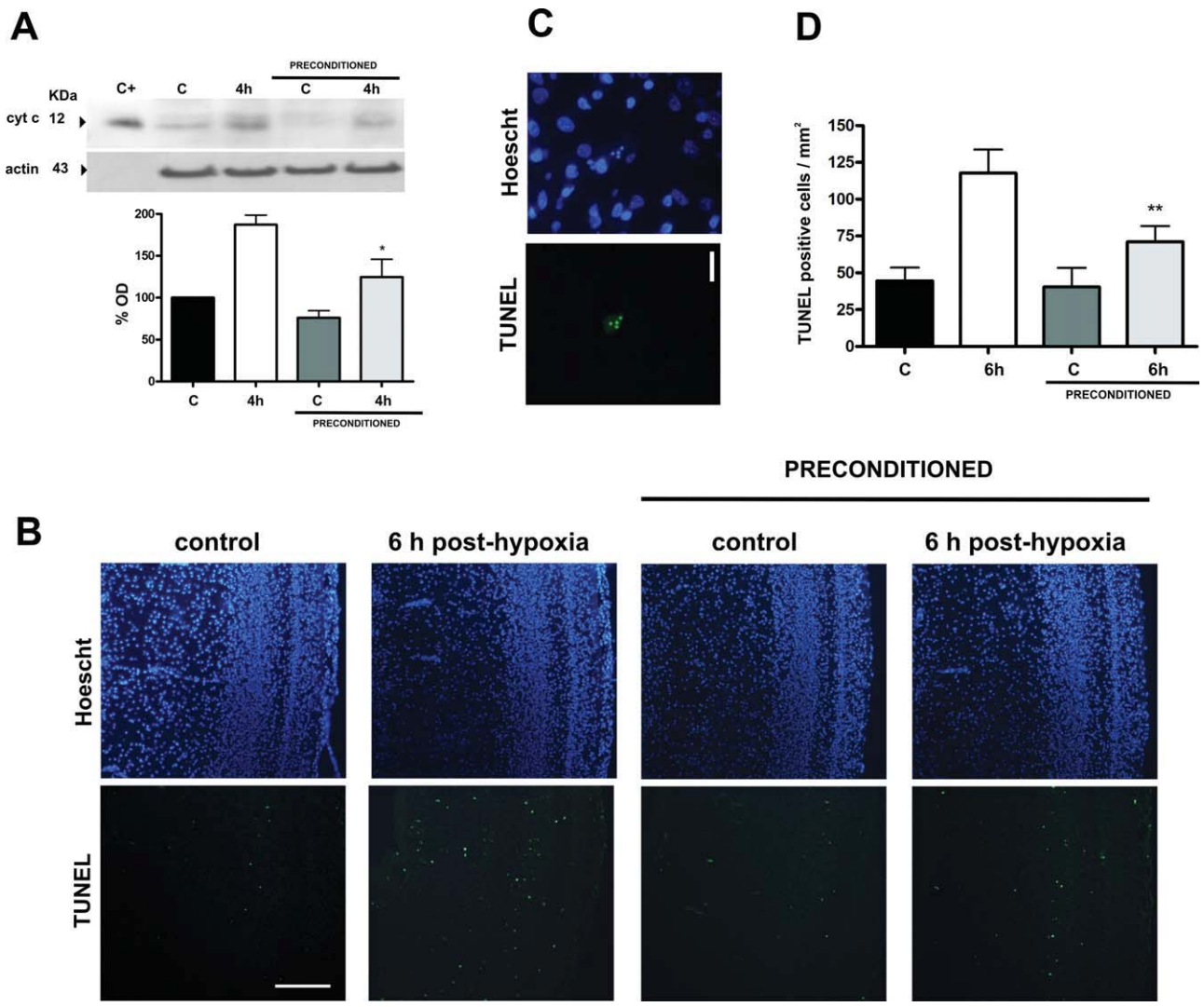

Fig. 2. Neuroprotective effects of hypoxic preconditioning. Embryos were preconditioned (8%O_2, 40 min) 24 hr before the hypoxic treatment (8%O_2, 60 min). Controls were not preconditioned. **A:** Representative immunoblotting of cytosolic fractions revealed with anticytochrome c antibody. Densitometric analysis is included. Membranes were stripped and reprobed with antiactin to confirm equal protein loading. C+, purified horse cytochrome c was used as a positive control. **B:** Photomicrographs showing representative Hoechst-stained and TUNEL-incubated optic lobe sections for each treatment. **C:** Detail showing TUNEL signal (green) colocalization with a pyknotic Hoechst stained nucleus (blue). Scale bar = 15 μm. **D:** Quantitative analysis is included. Values are means ± SEM of four independent experiments. Significantly different from non preconditioned posthypoxia value (⋆$P < 0.05$, ⋆⋆$P < 0.01$, ANOVA and Dunnett's posttest). Scale bar = 300 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The coadministration of ascorbate and Fe^{2+} was not used because it was found to be toxic for the embryos even at the lowest doses. This is due to the fact that the system Fe^{2+}/ascorbate is highly reactive, producing a burst of harmful hydroxyl radicals (Slivka, 1985).

HIF-1 Induction During Preconditioning Is Essential for the Development of Neuroprotection

Most importantly, we observed that the neuroprotective effects of hypoxic preconditioning could be mimicked by preconditioning with $CoCl_2$, preventing cytochrome c release and DNA fragmentation (Fig. 6A). Conversely, the neuroprotective effect was strongly attenuated when ascorbate or Fe^{2+} was administered before hypoxic preconditioning (Fig. 6C). Moreover, appropriate controls demonstrated that the administration of $CoCl_2$, Fe^{2+}, or ascorbate at ED 11 does not per se increase cytochrome c release (Fig. 6B) or DNA fragmentation (data not shown) in ED 12. Taken together, these results indicate that HIF-1 accumulation during preconditioning is essential for the achievement of neuroprotection.

DISCUSSION

Different experimental models have established that brief and moderate injuries can result in protective molecular and metabolic regulations now known under the

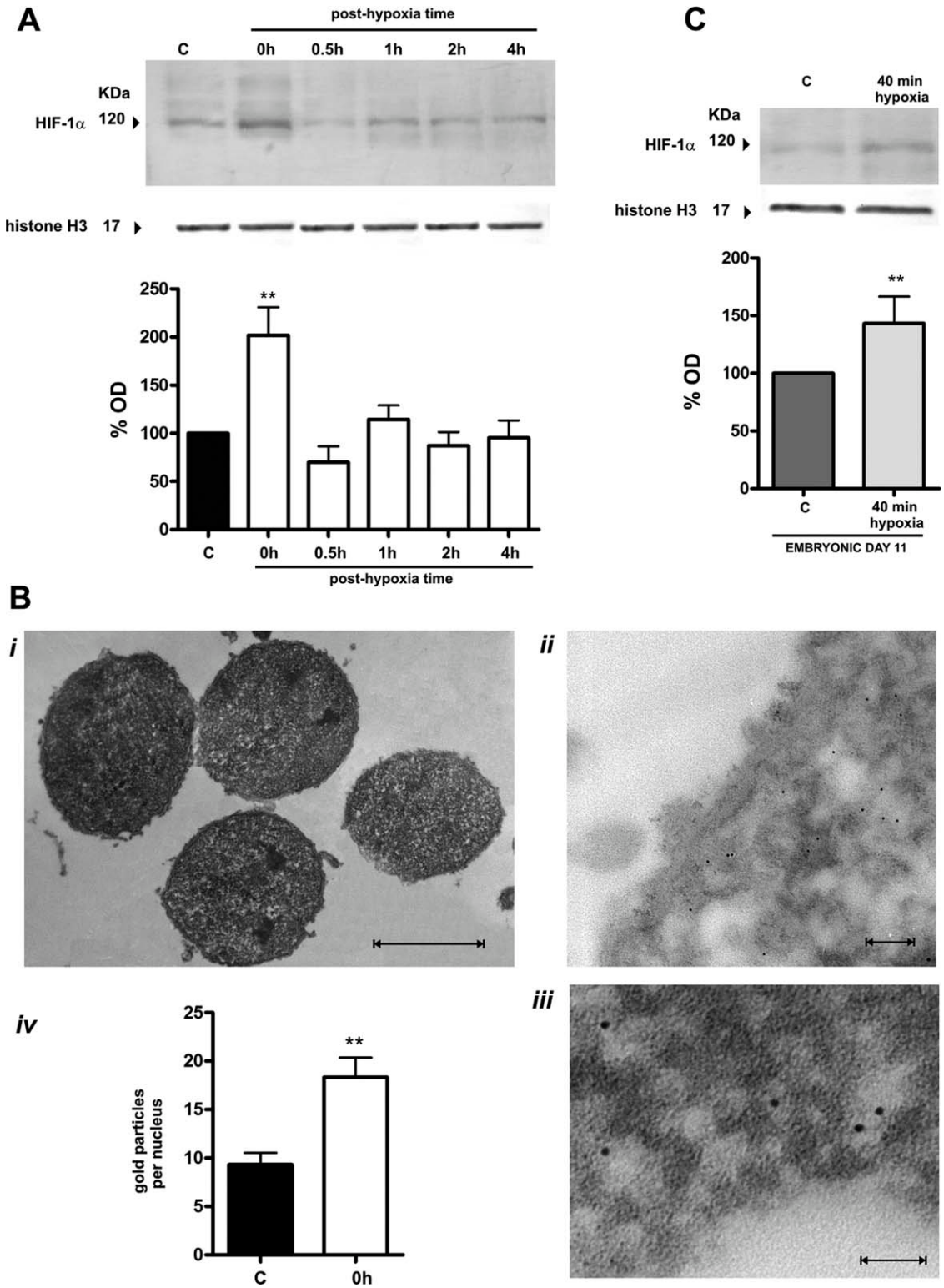

Fig. 3. HIF-1 is induced by hypoxic treatment. Embryos were subjected to hypoxia and sacrificed at different posthypoxia times. **A:** Representative immunoblotting of purified soluble nuclear fractions revealed with anti-HIF-1α antibody. Membranes were stripped and reprobed with antihistone H3 to confirm equal protein loading. Densitometric analysis of 0–4-hr kinetics is included. Values are means ± SEM of four independent experiments. Significantly different from control value (**$P < 0.01$, ANOVA and Dunnett's posttest). **B:** HIF-1α induction at 0 hr posthypoxia was confirmed by immuno-EM analysis of purified nuclei obtained from chick optic lobes. Samples were reacted with mouse antibodies specific for HIF-1α (10 nm gold). **i:** Representative sample of purified nuclei used for immuno-EM analysis. **ii,iii:** Detail of a nucleus showing gold immunolabeling. **iv:** Quantitative analysis is included. Fifty nuclei were analyzed in each condition (**$P < 0.01$, Student's *t*-test). **C:** Preconditioning-like hypoxic treatment on ED 11 induces per se HIF-1. Representative immunoblotting of purified soluble nuclear fractions revealed with anti-HIF-1α antibody. Membranes were stripped and reprobed with antihistone H3 to confirm equal protein loading. Values are means ± SEM of four independent experiments. Significantly different from control value (**$P < 0.01$, Student's *t*-test). Scale bars = 300 nm in i; 150 nm in ii; 50 nm in iii.

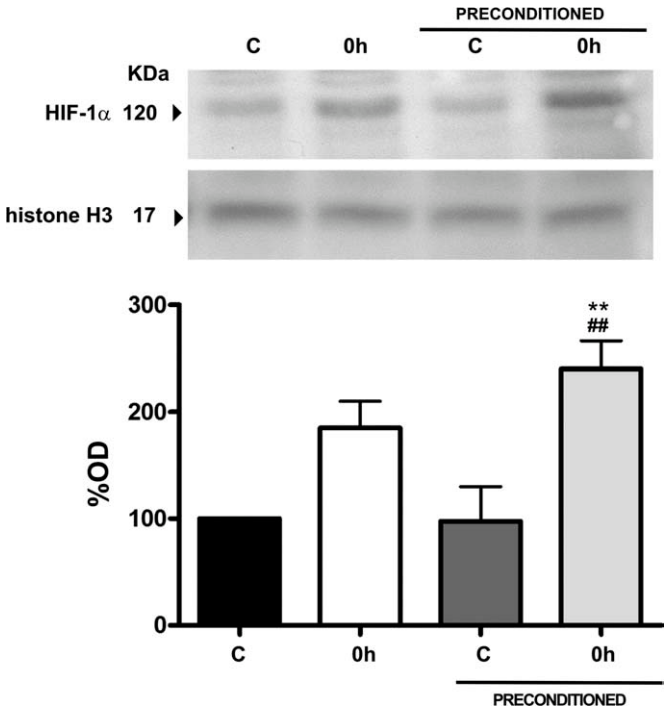

Fig. 4. Hypoxic preconditioning enhances HIF-1 accumulation after hypoxia. Representative immunoblotting of purified soluble nuclear fractions revealed with anti-HIF-1α antibody. Membranes were stripped and reprobed with antihistone H3 to confirm equal protein loading. Values are means ± SEM of four independent experiments. **Significantly different from control value, ##significantly different from the value at 0 hr posthypoxia ($P < 0.01$, ANOVA and Dunnett's posttest).

name of *preconditioning* (Chen and Simon, 1997). It is assumed that the initial exposure to stress stimulates several endogenous protective or preventive mechanisms, resulting in the development of tolerance. In particular, it has been demonstrated that preconditioning with mild hypoxia can protect the brain against subsequent hypoxic/ischemic injuries (Gidday et al., 1994; Ran et al., 2005). Similar studies have demonstrated the favorable effects of hypoxic preconditioning in adult rodents (Miller et al., 2001). In line with these results, here we have established an experimental prenatal model in which preconditioning with a low-intensity hypoxic exposure increases brain tolerance against a more severe hypoxic injury 24 hr later.

Usually, neuroprotective effects appear from 1 to 3 days after the preconditioning (Kitagawa et al., 1990). This is consistent with the fact that both hypoxic and ischemic preconditioning require synthesis of new RNA and protein, because hypoxia-induced tolerance is blocked by inhibitors of RNA and protein synthesis (Gage and Stanton, 1996). Therefore, an appealing hypothesis is that transcription factors are the central molecular mediators of the response. Accordingly, several transcription factors have been identified that are upregulated in the brain following hypoxia, including metal

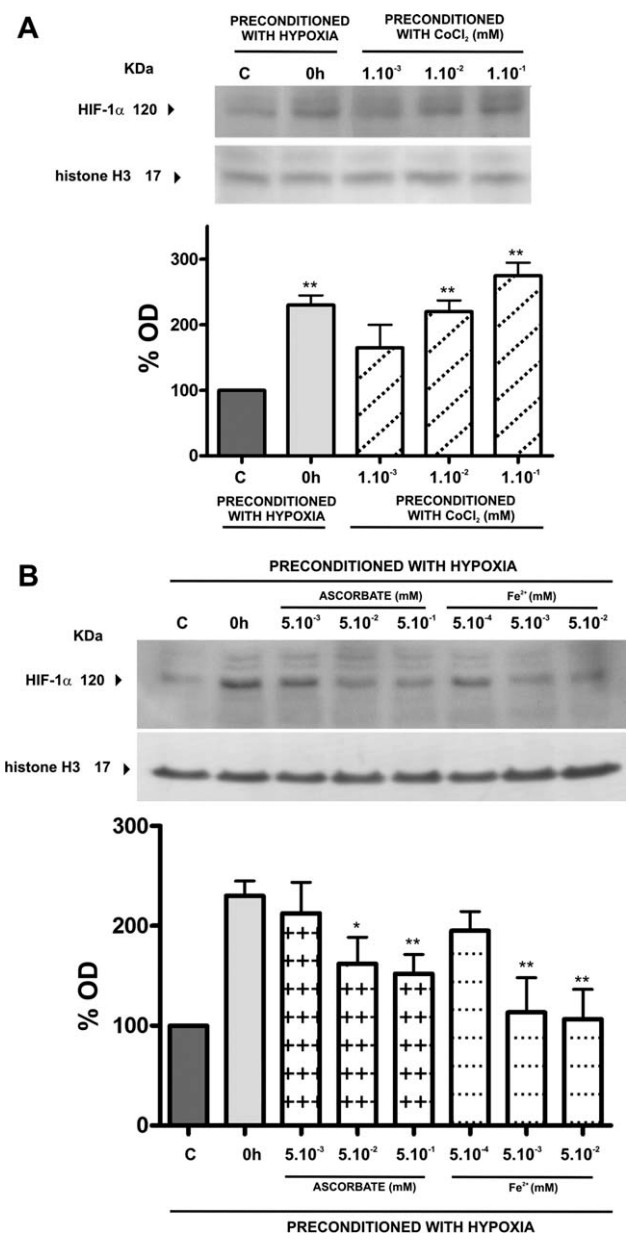

Fig. 5. HIF-1 accumulation after hypoxia is affected by previous stabilization and destabilization of HIF-1. Representative immunoblottings of purified soluble nuclear fractions revealed with anti-HIF-1α antibody. Membranes were stripped and reprobed with antihistone H3 to confirm equal protein loading. Values are means ± SEM of four independent experiments. **A:** Embryos were preconditioned with mild hypoxia (8%O_2, 40 min) or with increasing doses of $CoCl_2$, a chemical inducer of HIF-1, 24 hr before the hypoxic treatment (8%O_2 60 min). Significantly different from hypoxic-preconditioned control value (**$P < 0.01$, ANOVA and Dunnett's posttest). **B:** Embryos were preconditioned (8%O_2, 40 min) 24 hr before the hypoxic treatment (8%O_2, 60 min). Embryos were treated with Fe^{2+}, ascorbate, or vehicle (saline) 20 min before preconditioning. Fe^{2+} and ascorbate downregulate HIF-1 by stimulating HIF-prolyl hydroxylase activity. Significantly different from the value at 0 hr posthypoxia (*$P < 0.05$ or **$P < 0.01$, ANOVA and Dunnett's posttest).

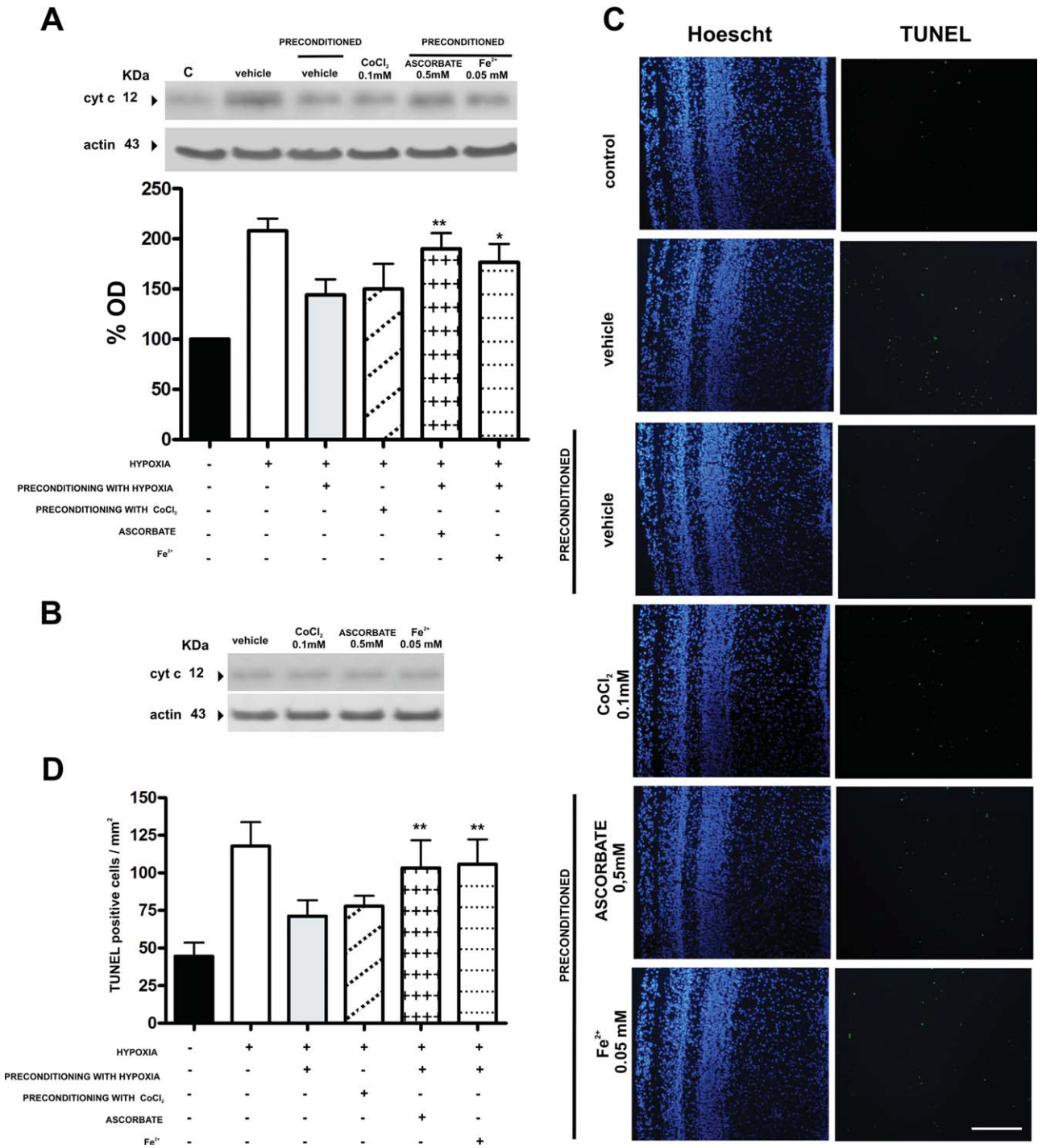

Fig. 6. Neurprotective effects of hypoxic preconditioning depend on HIF-1 accumulation. Embryos were preconditioned with mild hypoxia (8%O_2 40 min) or with $CoCl_2$ 24 hr before the hypoxic treatment (8%O_2 60 min). Embryos were treated with Fe^{2+}, ascorbate, or vehicle (saline) 20 min before hypoxic preconditioning. Control embryos were not subjected to the hypoxic treatment. **A:** Representative immunoblotting of cytosolic fractions revealed with anticytochrome c antibody. Densitometric analysis is included. Membranes were stripped and reprobed with antiactin to confirm equal protein loading. **B:** Embryos were treated with Fe^{2+}, ascorbate, or vehicle (saline) and then preconditioned (8%O_2 40 min) or preconditioned with $CoCl_2$ but not subjected to the hypoxic treatment on ED 12. **C:** Photomicrographs showing representative Hoechst-stained and TUNEL-incubated optic lobe sections for each treatment. **D:** Quantitative analysis is included. Values are means ± SEM of four independent experiments. Significantly different from vehicle-treated hypoxic-preconditioned value ($\star P < 0.05$ or $\star\star P < 0.01$, ANOVA and Dunnett's posttest). Scale bars = 300 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

transcription factor 1 (MTF-1), early growth response protein 1 (Egr-1), v-ets erythroblastosis virus E26 oncogene homolog 1 (avian; ETS1), nuclear factor-κB (NF-κB), activator protein 1 (AP1), and HIF (Bernaudin et al., 2002; Kenneth and Rocha, 2008). Among them, HIF is recognized as a key modulator of the transcriptional response to hypoxic stress. In support of this, several HIF target genes have been identified that are induced in hypoxia in the CNS and may contribute to neuroprotection. For example, it has been widely proved that HIF-1 guides a shift toward nonoxidative forms of carbon metabolism and ATP production by promoting the expression of glucose transporters, glycolytic enzymes, and LDHA, which replenish NAD^+ for further glycolysis (Gordan et al., 2007). Furthermore, the efficacy of several HIF targets, such as erythropoietin (EPO) and heme oxygenase 1 (HO-1), has been tested in animal and preclinical models of stroke (Lakkisto et al., 2009; Lundbeck, 2009).

However, other studies suggest that an acute increase in HIF-1 levels may imply a signal of severe hypoxic damage and result in cell death through the activation of another set target genes, such as proapoptotic members of the Bcl-2 family like BNIP-3 (Bruick, 2000; Sowter et al., 2001), p53, and caspases (Li et al., 2005). This dual role has also been observed for some target genes of HIF-1. For instance, whereas VEGF has been found to have neuroprotective properties under different conditions (Sun and Guo, 2005; Wang et al., 2006), other experimental approaches indicate that VEGF increases vascular permeability and inflammation, thus favoring cerebral edema (Lafuente et al., 2002; Althaus et al., 2006; Chi et al., 2007). In the same sense, a recent report has shown that administration of 2-methoxyestradiol and tricyclodecan-9-ylxanthogenate, two negative regulators of HIF-1, have neuroprotective effects in a model of focal ischemia induced by middle cerebral artery occlusion (MCAO; Chen et al., 2007). Controversial results have also been obtained with conditional knockout mice in which neuronal-specific lack of HIF-1α was achieved via Cre-lox technology. Helton et al. (2005) were the first to report an advantageous effect of neuronal HIF-1α knockdown in the bilateral common carotid artery occlusion (BCCAO) model of stroke. This was attributed to the suppression of proapoptotic HIF-1 target genes. A contrasting report from Baranova et al. (2007), who used the same transgenic lines, showed that HIF-1α knockdown was deleterious in the MCAO stroke model. Although the cause of the discrepancies between these reports remains unclear, they may be a reflection of the differences between stroke models used [global (BCCAO) vs. focal (MCAO) ischemia] and the duration of the ischemic insult (75 vs. 30 min). To explain these contrasting outcomes of HIF-1, leading to either prosurvival or prodeath effects, it has been proposed that different levels of hypoxia trigger divergent responses: moderate HIF-1 induction would facilitate ATP production under hypoxic conditions, whereas high induction would trigger the apoptotic machinery, eventually leading to cell death (Semenza, 2000; Chen et al., 2009). Altogether, these observations indicate that the consequences of HIF-1α stabilization must be determined in each experimental paradigm. In addition, different studies have shown consistent differences in HIF-1 accumulation kinetics between cells from different species (Wiesener et al., 2003) and in response to varying degrees of hypoxia (Bracken et al., 2006). In our experimental model, HIF-1 was found to be maximally increased at the end of the hypoxic treatment, and the amount of this accumulation was dependent on the duration of the hypoxic treatment. We found that preconditioning had a reinforcing effect on HIF-1 accumulation during the subsequent hypoxic injury.

This study has also demonstrated that HIF-1 induction during hypoxic preconditioning is necessary for the development of tolerance against a subsequent hypoxic injury. We used an experimental approach based on the positive and negative modulation of PHDs, the main regulators of HIF-1α. On the one hand, we stimulated PHDs activity 20 min before preconditioning by the in vivo administration of Fe^{2+} or ascorbate and observed the prevention of both the HIF-1 increase induced by preconditioning and its neuroprotective effects. In correspondence with this result, recent studies performed in cell cultures have shown that PHD activity is tightly regulated by Fe^{2+} and ascorbate levels (Pan et al., 2007). In a complementary approach, hypoxic preconditioning was substituted by $CoCl_2$ administration. We found that HIF-1 induced during the preconditioning phase, under normoxic conditions, reproduced HIF-1 intensification after the hypoxic injury and the development of tolerance. Therefore, our study confirms previous correlations between the protective effects of iron chelators and their ability to stabilize HIF-1 (Zaman et al., 1999). These results suggest that no other processes triggered by hypoxic preconditioning but HIF-1 accumulation play a main role in the development of tolerance. In line with this result, by using hypoxic preconditioning with neuronal-specific conditional knockout mice, it has recently been shown that HIF-1α in neurons is essential to obtain an improved brain tissue oxygenation after hypoxic injury (Taie et al., 2009). However, in this model, HIF-1 is absent not only during preconditioning but also during the hypoxic injury, so it is not possible to reveal the effects of HIF-1 on its own pathway. It is important to note the critical relevance, in the context of our study, of the use of a hypoxic nonischemic model to avoid local inflammation. Inflammation generates an increase in local vascular permeability, a reduced circulation, and an increased metabolic demand from infiltrating immune cells, eventually leading to a depletion of O_2 (Iadecola and Alexander, 2001) and therefore to a secondary wave of HIF-1 activation that would mask the effects of hypoxic preconditioning on this pathway.

As mentioned above, HIF-1 induction during preconditioning is essential to obtain an enhancement of HIF-1 accumulation during the hypoxic injury and neuroprotective effects. Nevertheless, it remains to be further

investigated whether HIF-1 enhancement observed after the hypoxic injury has a causal role in the hypoxic tolerance or are both independent effects of the preconditioning.

Importantly, our study also demonstrates that in vivo administration of Fe^{2+} and ascorbate modulates PHD activity and therefore HIF accumulation, indicating the exquisite sensitivity of these enzymes toward the changing levels of these fundamental molecules and the fact that these enzymes do rely on the labile pool of iron and ascorbate in the cells for their activity. This is particularly relevant in a context in which it has been proposed that PHDs could be targets for neuroprotection in the CNS, for the treatment of ischemic diseases, including stroke. Because the inhibition of PHDs activates the HIF response, it has been proposed that administration of PHD inhibitors could mimic, at least in part, the protective effects of exposure to hypoxia. In this regard, inhibition of PHDs with different compounds has previously been reported to stabilize HIF-1α in the brain and to ameliorate neuronal death after MCAO (Siddiq et al., 2005; Nagel et al., 2010). Although PHD inhibitors such as $CoCl_2$ may not be suitable candidate drugs for successful clinical translation, this study lends insight into the mechanisms underlying the concept of PHD inhibition for treatment of cerebral hypoxic injury and highlights the importance of assessing physiological variables in studies that investigate neuroprotection.

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