Hypoxic preconditioning protection is eliminated in HIF-1 α knockout mice subjected to neonatal hypoxia–ischemia

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BACKGROUND: Hypoxic preconditioning (HPc) protects the neonatal brain in the setting of hypoxia–ischemia (HI). The mechanisms of protection may depend on activation of hypoxia-inducible factor (HIF-1 α). This study sought to clarify the role of HIF-1 α after HPc and HI.

METHODS: To induce HPc, HIF-1 α knockout and wild-type (WT) mice were exposed to hypoxia at postnatal day 6. At day 7, the mice underwent HI. Brain injury was determined by histology. HIF-1 α , downstream targets, and markers of cell death were measured by western blot.

RESULTS: HPc protected the WT brain compared with WT without HPc, but did not protect the HIF-1 α knockout brain. In WT, HIF-1 α increased after hypoxia and after HI, but not with HPc. The HIF-1 α knockout showed no change in HIF-1 α after hypoxia, HI, or HPc/HI. After HI, spectrin 145/150 was higher in HIF-1 α knockout, but after HPc/HI, it was higher in WT. Lysosome-associated membrane protein was higher in WT early after HI, but not later. After HPc/HI, lysosome-associated membrane protein was higher in HIF-1 α knockout.

CONCLUSION: These results indicate that HIF-1 α is necessary for HPc protection in the neonatal brain and may affect cell death after HI. Different death and repair mechanisms depend on the timing of HPc.

Undetectable under normoxic conditions, the transcription factor, hypoxia-inducible factor (HIF-1 α), is activated and stabilized in response to hypoxia, suggesting that it has a role in response to oxidative injury and in hypoxic preconditioning (HPc) protection (1). In the neonatal brain, a prior episode of hypoxia can attenuate injury from subsequent hypoxia–ischemia (HI) (2), and the protection afforded by HPc is long lasting (3). Just as the mechanisms of neuronal cell death are complex (4), the mechanisms of HPc protection likely involve a complex cascade of cellular and molecular events. HPc has been shown to induce more than 1,000 genes in the P6 rat brain within the first 24h, predominantly genes involved in apoptosis and brain development (5). Hypoxia induces many of the genes regulated by HIF-1 α in the brain (6), and preconditioning with hypoxia induces HIF-1 α and promotes cell survival in the subsequently hypoxic or ischemic brain (1). The

role of HIF-1 α and its target genes, whether beneficial or detrimental, depends on such factors as severity and type of insult and age of the animal (7).

Of particular interest among the large number of target genes of HIF-1 α are vascular endothelial growth factor (VEGF) and erythropoietin as these have been shown to be induced by hypoxia (8,9) and to have protective properties in the brain under certain conditions (10–12), as well as to brain cells *in vitro* (13). VEGF administered intracerebroventricularly to neonatal rats after HI can decrease the severity of injury, in a dose-dependent manner (14), and exogenous erythropoietin shows beneficial effects, given either intracerebroventricularly (15) or systemically (12), after neonatal HI and stroke.

We have previously suggested that there is a protective role for HIF-1 α in neonatal HI because genetic reduction of HIF-1 α worsens HI brain injury (16). In order to explore the role of HIF-1 α in HPc, we used the same strain of mutant mice with a neuron-specific reduction of HIF-1 α and subjected these mice to a period of hypoxia, as a preconditioning stimulus, prior to HI. We measured histopathological brain injury and downstream regulators (VEGF and extracellular signal-regulated kinase (ERK)) and protein markers for cell death pathways (spectrin breakdown products for necrosis and apoptosis and lysosome-associated membrane protein (LAMP2) for autophagy) to better understand how brain injury is affected after HPc.

RESULTS

Histological Analysis for Degree of Injury

HPc was associated with significantly less histopathological damage in selectively vulnerable brain regions of the wild-type (WT) brain with HPc than WT without HPc (**Figure 1** and see **Supplementary Figure S1** online). (**Figure 1a**, median scores, WT-HI = 14 (range = 2–24), WT-HPc/HI = 5 (range 2–20), $P < 0.01$). The HIF-1 α -deficient brain, however, showed no protection with HPc (**Figure 1a**, median scores, HIF-1 α knockout-HI = 17 (range 3–24), HIF-1 α knockout-HPc/HI = 16 (range 5–24)). Significant protection in the WT-HPc/HI mouse brain was seen both in the cortex (**Figure 1b**, median scores, WT-HI = 6 (range 2–9), WT-HPc/HI = 3 (range 1–9), $P = 0.01$) and hippocampus (**Figure 1c**,

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Figure 1. Hypoxic-ischemic brain injury scores. Filled circles represent brains with hypoxia-ischemia (HI) alone, and open circles represent brains with hypoxic preconditioning (HPC) prior to HI. Solid horizontal line represents median score of mice receiving HI alone, and dashed line represents median score of mice receiving HPC prior to HI. **(a)** Overall score (range 0–24). HPC protected the wild-type (WT) brain from subsequent HI. WT-HI (median = 14, range 2–24) vs. WT-HPC/HI (median = 5, range 2–20), $P = 0.01$. **(b)** Cortex (range 0–9). WT-HI (median = 6, range 2–9) vs. WT-HPC/HI (median = 3, range 1–9), $P = 0.01$. **(c)** Hippocampus (range 0–12). WT-HI (median = 7, range 0–12) vs. WT-HPC/HI (median = 2, range 0–10), $P = 0.001$. The hypoxia-inducible factor (HIF-1 α) knockout brain was not protected by HPC. **(a)** HIF-1 α knockout-HI (median = 17, range 3–24) vs. HIF-1 α knockout-HPC/HI (median = 16, range 5–24). **(b)** HIF-1 α knockout-HI vs. HIF-1 α knockout-HPC/HI (both: median = 6, range 3–9). **(c)** HIF-1 α knockout-HI vs. HIF-1 α knockout-HPC/HI (both: median = 8, range 0–12).

median scores, WT-HI = 7 (range 0–12), WT-HPC/HI = 2 (range 0–10), $P < 0.001$). The median score of the cortex for HIF-1 α knockout-HI = 6 (range 3–9) and HIF-1 α knockout-HPC/HI = 6 (range 3–9). The median score of the hippocampus for HIF-1 α knockout-HI = 8 (range 0–12) and HIF-1 α knockout-HPC/HI = 8 (range 0–12).

Animal Numbers, Mortality, and Sex of Histology Group

For determination of brain injury by histological analysis, the total number of WT mice was 123; 56 (46%) underwent HPC. The total number of HIF-1 α knockout mice was 117; 53 (45%) underwent HPC (Table 1). Mortality occurred both during and after experimental procedures (Table 1). There were no differences in injury scores between male and female in each group (Table 1).

Protein Expression

HIF-1 α protein expression increased in WT, but not in HIF-1 α knockout cortex after HPC (Figure 2a). WT hypoxia is higher than WT naive at 15 min ($P < 0.02$), at 4 h ($P < 0.01$), and at 24 h ($P < 0.05$). HIF-1 α protein expression was also higher in WT naive compared with HIF-1 α knockout naive (Figure 2a, $P < 0.002$) and in WT hypoxia compared with HIF-1 α knockout-hypoxia at 15 min ($P < 0.02$) and at 4 h ($P = 0.001$). By 24 h after hypoxia, however, HIF-1 α expression in the WT has declined to levels similar to HIF-1 α knockout ($P < 0.10$). In mice subjected to HI (Figure 2b), HIF-1 α protein expression increased in WT early (15 min) compared with WT naive ($P < 0.002$) but was not changed significantly at later timepoints (4 or 24 h). WT is also higher than HIF-1 α knockout at 15 min ($P < 0.02$), but not at 4 or 24 h. HPC suppresses subsequent expression of HIF-1 α after HI (Figure 2c). Although no elevation of HIF-1 α was observed in WT cortex of HPC-/HI-treated brains when compared with naive, HIF-1 α expression was decreased in the HIF-1 α knockout cortex at 15 min compared with WT naive ($P < 0.05$).

VEGF protein expression (Figure 3) was not significantly elevated in either the WT or the HIF-1 α knockout cortex by hypoxia, HI, or HPC/HI. There were no significant differences in ERK activation, as shown by the phospho-ERK/ERK ratio, with hypoxia treatment alone, HI, or HPC/HI (Figure 4).

Spectrin was measured as a marker of the two major types of cell death (Figure 5). Necrotic cell death mediated via calpain is seen by the spectrin cleavage products at 145/150 kD, and apoptotic cell death mediated via caspase-3 by the spectrin cleavage products at 120 kD. There were no changes with hypoxia alone (data not shown). After HI, however, spectrin 145/150 was higher at 24 h in HIF-1 α knockout than WT (Figure 5a, $P < 0.04$). Spectrin 120 was not significantly changed in either WT or HIF-1 α knockout after HI, but the HIF-1 α knockout cortex showed a trend toward more spectrin 120 than WT at 24 h (Figure 5b, $P = 0.08$). After HPC/HI, spectrin 145/150 was threefold higher in WT than HIF-1 α knockout at 4 h (Figure 5c, $P = 0.002$). Also, both WT and HIF-1 α knockout showed increased spectrin 145/150 at 24 h compared with WT naive (Figure 5c, $P < 0.05$). Spectrin 120 was only

Table 1. Animal numbers, mortality, and sex

Number of mice and mortality, <i>n</i> (%) ^a	Manner of death, <i>n</i> (%) ^b							Sex, <i>n</i> (median score) ^c	
	Total	Survived	Died	HPc	W/dam	Hypoxia	Post-tx	Male	Female
WT-HI	67 (28)	32 (48)	35 (52)	NA	6 (17)	22 (63)	7 (20)	17 (12)	15 (15)
WT-HPc/HI	56 (23)	35 (63)	21 (37)	1 (5)	5 (24)	9 (43)	6 (29)	16 (8)	19 (6)
KO HI	64 (27)	37 (58)	27 (42)	NA	4 (15)	17 (63)	6 (22)	12 (15)	25 (18)
KO HPc/HI	53 (22)	33 (62)	20 (38)	7 (35)	3 (15)	5 (25)	5 (25)	15 (15)	18 (17)
Total	240	137 (57)	103 (43)						

HI, hypoxia-ischemia; HPc, hypoxic preconditioning; KO, knock out; tx, treatment; WT, wild type.

^aTotal number of mice (*n*) and (%) per treatment group. Total number (*n*) and (%) that survived to perfusion at P12 or died prior to P12. ^bManner of death for those that died before perfusion: during HPc; with dam after surgery; during postsurgery hypoxia treatment; after all treatments. ^cTotal number of male and female mouse brains per experimental group scored for degree of injury and median score for each group.

significantly higher in WT at 24 h after HPc/HI compared with WT naive (Figure 5d, $P < 0.05$).

After HI, LAMP2, a marker of autophagy, was greater in WT compared with HIF-1 α knockout at 15 min (Figure 6a, $P < 0.0002$), but declined by 4 h and remained low at 24 h compared with WT naive ($P < 0.02$). After HPc/HI, LAMP2 was not significantly changed in WT compared with naive. In the HIF-1 α knockout, however, LAMP2 increased at 15 min (Figure 6b, $P < 0.03$), again at 4 h ($P < 0.002$) and remained elevated at 24 h ($P < 0.03$). The HIF-1 α knockout also had greater LAMP2 than corresponding WT with HPc/HI at 15 min ($P < 0.05$) and at 4 h ($P < 0.02$), but not at 24 h.

DISCUSSION

This study supports the hypothesis that HIF-1 α plays an important role in the pathophysiology of neonatal HI brain injury and is a necessary component of the protective mechanisms involved in HPc. We have previously shown that HIF-1 α knockout mice have more severe injury than their WT littermates after HI, suggesting a protective role for HIF-1 α (16). Similarly, in this study, the HIF-1 α knockout mice again have higher median injury scores than WT littermates. The finding that HPc attenuates HI injury in the WT brain, while the HIF-1 α -deficient brain is strikingly resistant to protection by HPc, suggests that HPc-induced stabilization of HIF-1 α is an important factor in subsequent protection from HI injury.

In a study of adult stroke using the same strain of conditional, neuron-specific HIF-1 α knockout mouse, investigators found increased brain injury in the HIF-1 α -deficient mice compared with WT (17). However, these investigators found that HPc was protective to both the WT and HIF-1 α knockout adult mouse brain subjected to ischemia. This disparity from what we report in the neonatal brain suggests there are different mechanisms involved in preconditioning protection, at least in part, between immature and mature mice. This is not surprising given the selective vulnerability of the developing brain to injury, particularly injury associated with oxidative stress (18). This vulnerability may be due, in part, to the finding that the developing brain accumulates more of the oxidant H₂O₂ after HI than the adult mouse brain after HI (19). HPc is known to initiate activation of endogenous antioxidants in response to oxidative stress (6,18) along with HIF-1 α . It is, therefore,

likely that H₂O₂ modulates the stabilization and transcriptional activation of HIF-1 α to regulate a number of protective genes that are responsible not only for oxygen homeostasis but also for brain protection and repair after severe oxidative stress in neonatal HI. Although overexpression of the endogenous antioxidant glutathione peroxidase-1 reduces HI injury (20), HPc reverses this protection (21). This reversal of protection with HPc is associated with an increase in H₂O₂ accumulation after HI in the glutathione peroxidase-1-overexpressing mice to WT levels by 24 h. This suggests that although H₂O₂ at high concentrations is a mediator of cell death, it may also play a part in HPc protection at low levels, particularly during the early phase of the injury process. The accumulation of H₂O₂ in the neonatal brain exposed to HI as a consequence of incomplete deactivation by glutathione peroxidase-1 (19) is toxic to neurons in high concentrations, but it has also been associated with protection at lower levels (22). Exogenous H₂O₂, at low concentrations, acts as a preconditioning agent by inducing HIF-1 α in neurons *in vitro* and conferring protection from subsequent ischemia (22). Micromolar levels of H₂O₂ *in vitro* can block HIF-1 α degradation (23). Endogenous H₂O₂ produced by HPc in superoxide dismutase-transgenic neurons *in vitro* induces HIF-1 α and is protective against subsequent extended hypoxia (24).

High levels of H₂O₂ are known to stimulate necrosis and apoptosis, whereas low levels induce an antiapoptotic program (25). We recently showed that although HIF-1 α expression in these glutathione peroxidase-1-overexpressing mice was similar to WT after a period of hypoxia, ERK1/2 activation was prevented (26). The WT mice had a transient increase in ERK at 30 min, not at 0 min or 6 h. Our observation that HIF-1 α knockout mice do not benefit from HPc, therefore, may indicate aberrant H₂O₂ signaling as well as ERK1/2 activation in these animals. Although the ERK1/2 pathway has been suggested to be important for the regulation of HIF-1 α after neonatal HI (27), we did not observe significant alterations in ERK signaling. This disparity may be due to the different background strain used in our previous study (CD1) because differences in a number of transcription factors, including HIF-1 α and VEGF, have been shown between neonatal CD1 and C57Bl/6 mice (28). The interaction of HPc, HIF-1 α , and varying levels of H₂O₂ over time after injury merits further study.

Recently, it was shown in adult mice of a different strain of HIF-1 α knockout than that used in this study, that WT mice which received HPC prior to a subsequent hypoxic episode showed improved brain tissue oxygenation along with upregulation of inducible nitric oxide synthase, whereas the HIF-1 α knockout counterparts had no change in these parameters with identical hypoxia preconditioning (29). Also in adult mice, ischemic preconditioning of the heart has been shown to be lost in mice with a partial deficiency of HIF-1 α , along with a reduction in mitochondrial reactive oxygen species

production (30), indicating a potential role for reactive oxygen species in the mechanisms of ischemic preconditioning.

Despite its vulnerability, the immature brain may also have a unique ability for regeneration and repair. For example, immature (P9) rats show greater neurogenesis in response to HI than do juvenile (P21) rats (31). It has been shown that the target gene *VEGF* is induced by HPC and has a protective effect (11,14,32). However, we did not find significant alterations in expression of VEGF with HPC, suggesting that perhaps other HIF-1 target genes are involved in the protective mechanisms of HPC. Manipulation of the endogenous forms of VEGF for protection and repair merits further investigation (33).

We have previously shown that both degree of injury and mortality are affected by the strain of mouse subjected to HI (34). C57Bl/6, the background strain used in this study, has high mortality while being somewhat resistant to injury compared with outbred strains. Li *et al.* (28) also found high mortality with chronic hypoxia in P7 and P21 mice of the same background strain (C57Bl/6). For this study, we decreased the duration of hypoxia in order to limit mortality while providing a moderate degree of injury.

The finding that HIF-1 α is more strongly upregulated by hypoxia alone than by HI has also been shown in neonatal rats, and cleaved caspase-3 expression was correspondingly lower in the hypoxia-treated rats than the HI-treated rats, suggesting that higher levels of HIF-1 α may be associated with lower levels of apoptosis (33). This corresponds with our finding that spectrin 145/150 increased 4 h after HPC/HI in the WT brain, but not in the HIF-1 α knockout brain, along with no difference at 24 h, supporting the idea that there is a shift toward earlier cell death of a calpain-mediated nature in the WT, but not in the HIF-1 α knockout. In addition, spectrin 120 increased in WT at 24 h, suggesting a greater role for early, necrotic, calpain-specific cell death in the WT cortex with HPC/HI in addition to later, apoptotic cell death. It does not explain cell death in the HIF-1 α knockout, however. Perhaps other cell death mechanisms are involved, such as autophagy.

Autophagy has long been considered as another mechanism of cell death because it is upregulated in response to injury, but recent reports suggest it may have a protective role

Figure 2. Hypoxia-inducible factor (HIF-1 α) protein expression. Wild-type (WT) naive have approximately twice the HIF-1 α than HIF-1 α knockout naive (** $P < 0.002$). (a) Mouse cortex after hypoxia only. In the WT mouse brain exposed to hypoxia, HIF-1 increased at 15 min (* $P < 0.02$), at 4 h (* $P < 0.01$), and at 24 h (* $P < 0.05$) compared with WT naive. In addition, HIF-1 α is elevated in the WT brain compared with HIF-1 α knockout brain at 15 min (* $P < 0.02$) and at 4 h (** $P = 0.001$). HIF-1 α does not change with hypoxia treatment in the HIF-1 α knockout mice compared with HIF-1 α knockout naive at any timepoint. (b) Mouse cortex after HI. In the WT mouse brain exposed to HI, HIF-1 α increased at 15 min compared with naive (** $P < 0.002$). In addition, HIF-1 α is elevated in the WT brain compared with HIF-1 α knockout brain at 15 min (* $P < 0.02$). HIF-1 α does not change after HI in the HIF-1 α knockout cortex compared with naive at any timepoint. (c) Mouse cortex after hypoxic preconditioning (HPC) and HI. HIF-1 α does not change in the WT with HPC/HI. In the HIF-1 α knockout, HIF-1 α is decreased at 15 min compared with WT naive (* $P < 0.05$). White columns are WT. Black columns are HIF-1 α knockout. OD, optical density.

Figure 3. Vascular endothelial growth factor (VEGF) protein expression. (a) There were no changes in VEGF levels with hypoxia alone in either wild type (WT) or hypoxia-inducible factor (HIF-1 α) knockout. (b) There also were no significant changes in VEGF levels after hypoxia-ischemia (HI), although there was a trend toward an increase in the HIF-1 α knockout 15 min after HI ($P = 0.11$). (c) There was no change in VEGF expression in hypoxic preconditioning/HI mouse cortex in either WT or HIF-1 α knockout. White columns are WT. Black columns are HIF-1 α knockout.

in some circumstances (35). In neonatal HI, HPc has been shown to upregulate the autophagy marker Beclin-1 (36). However, our results with the autophagy marker, LAMP2 indicating increased autophagy at 4 and 24 h after HPc/HI in HIF-1 α knockout but not in WT, may correspond with a switch of cell death phenotypes and the lack of HPc protection seen in the HIF-1 α knockout. Again, the role of autophagy

Figure 4. Extracellular signal-regulated kinase (ERK) activation. Bar graph summarizes the ratio of phospho-ERK (pERK) to ERK compared with wild-type (WT) naive; there were no significant changes. (a) After hypoxia. There was a trend toward an increase in the WT cortex at 15 min ($P = 0.09$). (b) After hypoxia-ischemia (HI). There were trends toward an increase in HIFKO cortex at 15 min ($P < 0.14$) and at 4 h ($P < 0.09$). There was a trend toward a decrease in WT at 15 min ($P < 0.06$). (c) After hypoxic preconditioning/HI. There was a trend toward a decrease in the WT cortex at 15 min ($P = 0.06$). White columns are WT. Black columns are hypoxia-inducible factor-1 α knockout.

in neurodegeneration after neonatal HI is affected by severity, time and region and merits further investigation, especially with more autophagy markers.

Figure 5. Spectrin protein expression. (a) Spectrin 145/150 after hypoxia-ischemia (HI) is higher in hypoxia-inducible factor (HIF-1 α) knockout than wild type (WT) at 24 h (* P < 0.04). (b) Spectrin 120 after HI. There were no significant changes in spectrin 120. (c) Spectrin 145/150 after hypoxic preconditioning (HPC)/HI is elevated threefold in WT compared with HIF-1 α knockout at 4 h (** P = 0.002) and is also elevated in both WT and HIF-1 α knockout at 24 h compared with WT naive (* P < 0.05 for both). (d) Spectrin 120 after HPC/HI was elevated in WT at 24 h compared with WT naive (* P < 0.01). White columns are WT. Black columns are HIF-1 α knockout.

Figure 6. Lysosome-associated membrane protein (LAMP2) expression. (a) LAMP2 after hypoxia-ischemia (HI) was greater in wild type (WT) compared with hypoxia-inducible factor (HIF-1 α) knockout at 15 min († P < 0.0002), but declined by 4 h and remained low at 24 h compared with WT naive (* P < 0.02). (b) After hypoxic preconditioning (HPC)/HI, LAMP2 was not significantly changed in WT compared with naive. In the HIF-1 α knockout, however, LAMP2 increased at 15 min (* P < 0.03), again at 4 h (* P < 0.02) and remained elevated at 24 h (* P < 0.03) compared with WT naive. The HIF-1 α knockout also had greater LAMP2 than corresponding WT with HPC/HI at 15 min (* P < 0.05) and at 4 h (* P < 0.02), but not at 24 h. White columns are WT. Black columns are HIF-1 α knockout.

It is well known that HPC not only affects neurons, but other brain cells, which may act to protect neurons. In particular, the HPC of astrocytes may be an important factor in neuroprotection under certain circumstances (37). Sen *et al.* (38), e.g., have shown that HPC induces the precocious maturation

and differentiation of astrocytes, with a concomitant increase in their neuroprotective functions.

In summary, the hypoxic-ischemic neonatal HIF-1 α knockout mouse brain is not protected by hypoxia preconditioning, supporting our hypothesis that activation and modulation of

HIF-1 α is a critical component in the protective mechanisms of HPc in the neonatal brain. A greater understanding of the mechanisms of preconditioning protection in the neonatal brain may lead to more efficacious therapies aimed at protecting the human neonatal brain from HI injury.

METHODS

Animal Procedures

All animal research was approved by the University of California, San Francisco Institutional Animal Care and Use Committee and was performed with the highest standards of care under the US National Institutes of Health guidelines. Mice with conditional neuron-specific inactivation of HIF-1 α were generated using Cre/Lox technology. Adult mice of this strain have previously been well characterized (17,39). Briefly, the deletion was attained by breeding mice that have loxP-containing HIF-1 α alleles with “R1ag#5” line (40) mice expressing Cre recombinase under the control of the calcium/calmodulin-dependent kinase II promoter. The resulting litters produced mice with a forebrain predominant, neuron-specific deletion of HIF-1 α (HIF-1 α knockout), as well as littermates without the deletion (WT). Genotyping was carried out using PCR on tail DNA samples using standard methods. All mice negative by PCR for the Cre gene were considered WT.

Hypoxic Stimulus

At postnatal day 6 (P6), pups were placed in chambers maintained at 37 °C by a circulating water bath and subjected to 1 h of 8% oxygen (balance nitrogen). Naive pups were in a similar chamber exposed to room air or remained with the dam. Sham animals have been shown in previous experiments (16) to behave as the naïve, so in an effort to avoid unnecessary sacrifice of large numbers of animals, shams were omitted.

HI and Histology

At P7, pups ($n = 240$; 123 WT and 117 HIF-1 α knockout) underwent the Vannucci procedure of neonatal HI (41). Briefly, under isoflurane anesthesia, the right common carotid artery was permanently ligated. Following a 90-min recovery period with the dam, pups were exposed to hypoxia for 30 min (8% oxygen, balance nitrogen) while being maintained at 37 °C. We have previously shown that the background mouse strain, C57Bl/6, while somewhat resistant to injury from HI, has high mortality after HI (34). For determination of degree of injury, 5 d after HI pups were perfused intracardiac with cold 4% paraformaldehyde in 0.1 mol/l phosphate buffer, brains were removed and postfixed overnight in the same fixative followed by cryoprotection with 30% sucrose. Brains were sectioned on a Vibratome (50 μ m), and alternate sections were collected for Nissl staining with Cresyl Violet and Perl's iron stain to measure iron deposition. Brains were analyzed for degree of injury using a scoring system that employs both stains, as previously described (34), where 0 = no injury and 24 = severe injury with cystic infarction. With 30 min of hypoxia, we were able to limit mortality, while producing a moderate degree of injury in a majority of animals.

Western Blot Analysis

An additional cohort of animals ($n = 79$; 41 WT and 38 HIF-1 α knockout) underwent HPc and HI as previously described, and cortical brain samples were collected for determination of protein expression by western blot for HIF-1 α , VEGF, ERK 1/2 HIF-1 α knockout cortices were collected from three treatment groups: HPc alone ($n = 3$ at each timepoint), HI alone ($n = 3$ for 15 min, $n = 3$ for 4 h, $n = 6$ for 24 h), and HPc/HI ($n = 5$ for 15 min, $n = 5$ for 4 h, $n = 8$ for WT 24 h and $n = 7$ for HIFKO 24 h), as well as naive WT ($n = 13$) and naive HIF-1 α knockout ($n = 12$). Samples were snap frozen and stored at -80 °C until use, at which point nuclear and cytoplasmic fractions were prepared from the injured cortices using the Nuclear and Cytoplasmic Extraction Reagents (NE-PER; Pierce Biotechnology, Rockford, IL) according to the manufacturer's protocol. Briefly, tissue was homogenized in 250 μ l ice-cold CER I buffer with protease and phosphatase inhibitors. After incubation with CER II buffer, the

sample was centrifuged for 5 min at 16,000g at 4 °C and the supernatant served as the cytoplasmic extract. The pellet was resuspended in 90- μ l ice-cold NER buffer, vortexed, and centrifuged at 16,000g at 4 °C for 10 min. The supernatant was saved as the nuclear extract. The cytoplasmic and nuclear protein aliquots were stored at -80 °C until use. Protein concentrations were measured by BCA assay (Pierce), using bovine serum albumin as the standard.

Fifty micrograms of nuclear or cytoplasmic protein was applied to 4–12% bis-tris(hydroxymethyl)aminomethane sodium dodecyl sulfate polyacrylamide gels (Invitrogen, Carlsbad, CA) for electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline with 0.05% Tween 20 for 1 h at room temperature. The membranes were probed with the following primary antibodies overnight at 4 °C: HIF-1 α (Novus Biologicals, Littleton, CO); VEGF (Abcam, Cambridge, MA); ERK and phospho-ERK (Cell Signaling); spectrin (Millipore, Billerica, MA); LAMP2 (Abcam); and β -actin (Santa Cruz Biotechnology, Santa Cruz, CA). Appropriate secondary horseradish peroxidase-conjugated antibodies (1:2000; Santa Cruz Biotechnology) were used and signal was visualized with enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Image J software was used to measure the optical densities and areas of the protein signal on radiographic film after scanning. The optical density of the protein bands was normalized to β -actin. All experiments were repeated at least three times to ensure reproducibility of results.

Statistical Analysis

For histological scoring of brain injury, two-way ANOVA with Bonferroni posttest was used to determine interaction based on genotype. Subsequently, significance was determined by one-way ANOVA with Mann–Whitney test for multiple groups or *t*-test for two groups. For the western blots, one-way ANOVA with the Dunnett's test or *t*-test was used. Significance was set at $P < 0.05$. Analysis was done with Prism 5.0 (GraphPad Software, San Diego, CA).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/pr>

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