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HIF1α Signaling in the Endogenous Protective Responses after Neonatal Brain Hypoxia-Ischemia

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Keywords
Brain · Development · Hypoxia/ischemia · Hypoxia-inducible factor 1α

Abstract
Hypoxia-inducible factor 1α (HIF1α) is a key regulator of oxygen homeostasis, and its target genes mediate adaptive, protective, and pathological processes. The role of HIF1α in neuronal survival is controversial and the brain maturation stage is important in determining its function in brain ischemia or hypoxia-ischemia (HI). In this study, we used neuron-specific HIF1α knockout mice at postnatal day 9 (P9), and immature cortical neurons (days 7–8 in vitro) treated with the HIF1α inhibitor 2-methoxyestradiol (2ME2) or stabilizer dimethyloxalylglycine (DMOG), to examine the function of neuronal HIF1α in neonatal HI in vivo (Vannucci model) and in vitro (oxygen glucose deprivation, OGD). Inhibition of HIF1α with 2ME2 in primary neurons or deletion of neuronal HIF1α in P9 mice increased both necrotic and apoptotic cell death following HI, as evaluated by the protein levels of 145/150-kDa and 120-kDa spectrin breakdown products 24 h after HI. DMOG attenuated neuronal death right after OGD. Acute pharmacological manipulation of HIF1α synchronously regulated the expression of its targets, vascular endothelial growth factor (VEGF) and erythropoietin (Epo), in the same manner. The in vivo findings agree with our previous data using the same HIF1α-deficient mice at an earlier age. This study confirms the role of neuronal HIF1α signaling in the endogenous protective responses following HI in the developing brain.
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Introduction

Hypoxic-ischemic encephalopathy (HIE) is an important complication at birth and a significant cause of neonatal death. Moderate to severe HIE is usually associated with poor neurological outcomes including motor impairment or cognitive deficits [1]. Strategies that boost endogenous brain protection or stimulate regenerative repair hold the promise of translating research into clinical practice. One such strategy is targeting the transcription factor hypoxia-inducible factor 1 (HIF1), which plays a critical role in regulating cellular adaptation to hypoxia and oxidative stress [2–4]. HIF1 consists of 2 subunits, HIF1α and HIF1β. HIF1β does not respond to

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changes in oxygen tension, while the stability, subcellular localization, and transcriptional activity of HIF1α are especially affected by the oxygen level [5, 6]. In normoxic conditions, HIF1α is rapidly degraded by the prolyl hydroxylase (PHD)-dependent proteasomal system, but under hypoxia HIF1α protein degradation is prevented [7, 8]. Upon its accumulation and phosphorylation, HIF1α dimerizes with HIF1β, translocates into the nucleus, and promotes the transcription of hypoxia response element (HRE)-containing target genes that are involved in energy metabolism, redox homeostasis, erythropoiesis, vascular remodeling, and other processes [9, 10].

Induction of HIF1α has been reported in the ischemic penumbra in adult rats and mice, as well as in the neonatal brain following hypoxia-ischemia (HI) [11–15]. The role of HIF1α in mediating protective or injury mechanisms remains controversial, as it has been shown that HIF1α upregulates both pro- and antisurvival genes [16–19]. The discrepancies are related to the model of brain injury, the timing and duration of HIF1α stimulation or inhibition, the pathways that are activated downstream of HIF1α, the cell type in which HIF1α is induced, and the intercellular communication to transmit the HIF1α signaling. Selective loss of HIF1α function in astrocytes leads to different outcomes of neuron-specific inactivation of HIF1α [20]. Using a Cre-loxP-mediated conditional knockout approach, we have shown that deficiency in forebrain neuronal HIF1α caused more severe cortical injury than in wild-type (WT) littermates without HIF1α deletion [21]. However, the underlying mechanisms are not clear.

In this study, to further dissect out the specific contribution of HIF1α in neuronal cell death/survival, we used pharmacological strategies with a known HIF1α inhibitor 2-methoxyestradiol (2ME2), or a PHD inhibitor dimethyloxalylglycine (DMOG) that stabilizes HIF1α, to manipulate HIF1α levels in immature primary cortical neurons. We investigated whether acute manipulation of HIF1α expression has any impact on neuronal viability, as well as its effects on the HIF1α substrates, vascular endothelial growth factor (VEGF) and erythropoietin (Epo), both of which are proven to be beneficial in brain repair in rodent models of neonatal HI and neonatal stroke [22–26].

The maturation stage of the brain at the time of the HI insult is another important contributor to determine injury evolution, recovery, and repair. Our previous work showed that HIF1α was upregulated in postnatal day 7 (P7) mice following neonatal HI [21, 27], in P10 rats in a neonatal stroke model [28], and after treatment with desferoxamine (an iron chelator that could stabilize HIF1α) in neonatal stroke [29], or in primary hippocampal cultures undergoing oxygen glucose deprivation (OGD), an in vitro paradigm of HI [30]. HIF1α-deficient mice had increased brain injury at P7 in response to HI [21]. At the same age, hypoxic preconditioning alleviated brain damage in the WT mice, but not in the HIF1α knockout (KO) mice [27]. These studies support the notion that HIF1α plays a role in endogenous protective mechanisms and is important for hypoxic preconditioning in the neonatal brain. The P7 mouse brain is equivalent to the human brain late preterm, while the P9–P10 brain more adequately mimics the brain of full-term human infants and is therefore more relevant for studies of HIE. We chose to use P9 neuron-specific HIF1α KO mice for the HI model in this study to confirm the in vitro data and the results obtained with P7 mice.

Materials and Methods

C57BL/6 mice at embryonic day 15 (Charles River Laboratories Inc., Wilmington, MA, USA) were used for primary cortical neuronal culture. The mice that carry homozygous loxP-flanking exon 2 of HIF1α alleles were bred with heterozygous mice expressing Cre recombinase under the control of the calcium/calmodulin-dependent kinase II α (CaMKIIα) promoter to produce mice with a forebrain-predominant, neuron-specific deletion of HIF1α, as well as their WT littermates without the deletion [31–34]. All mice showing negative on PCR for the *Cre* gene were considered WT. Both sexes were used for neonatal HI procedure at P9.

Neonatal Brain HI Injury

Neonatal HI was performed as previously described with the Vannucci procedure [35]. At P9, the pups were anesthetized with isoflurane (2–3% isoflurane/balance oxygen) and the left common carotid artery was electrocauterized. The animals were allowed to recover for 1 h with their dam and were then exposed to 60 min of hypoxia in a humidified chamber at 37 °C with 10% oxygen/balance nitrogen. Sham-operated control animals received isoflurane anesthesia and exposure of the left common carotid artery, without electrocauterization or hypoxia. HI and sham animals were returned to their dams until they were euthanized.

Primary Cortical Neuronal Culture

Brains were removed from embryonic day 15 (E15) C57BL/6 mice [36] and maintained in high-glucose DMEM with 25 μM of 2-mercaptoethanol at 4 °C during dissection. Cortices were dissected and dissociated in trypsin (0.05% with EDTA) and DNAse (10 μg/mL) and then resuspended in neurobasal (NB) medium with 2% B27 and 1mM glutamine supplement (Gibco, Rockville, MD, USA). The cells were plated onto poly-D-lysine-precoated 96-well plates or 35-mm dishes (Corning Life Sciences, Tewksbury, MA, USA) at a density of 1×10^5 cells/cm^2. The cultures

were kept at 37 °C in a humidified incubator with a 5% CO_2 atmosphere. Three days after plating, half of the medium was replaced with the NB medium with 5-fluoro-2'-deoxyuridine (4 μg/mL) and uridine (20 μg/mL) to inhibit growth of nonneuronal cells. The neurons were used at 7–8 days in vitro for OGD experiments.

OGD and Drug Treatment

Briefly, the medium was washed out twice and replaced with NB medium without glucose (UCSF Cell Culture Facility) supplemented with B27/glutamine. Cultures were transferred to a humidified modular incubator chamber (Billups-Rothenberg Inc., Del Mar, CA, USA) and flushed with a gas mixture consisting of 95% N_2/5% CO_2 for 5 min. The chamber was then sealed and kept at 37 °C in an incubator for 6 h. The oxygen concentration was maintained at 0.3–0.5%, monitored by an oxygen analyzer (MSA medical products, Pittsburgh, PA, USA) throughout the experiment. OGD was ended by changing the medium to regular NB medium with glucose and returning the cells to a normoxic incubator. The control cells underwent the same medium change but with regular NB medium and were placed in a normoxic incubator [36]. 2ME2 (Sigma-Aldrich Corp, MO, USA) was constituted in DMSO as a stock solution, and further diluted in the culture medium to a final concentration of 50 μM. DMSO was diluted in the same manner to a final concentration of 0.5% as the vehicle control. DMOG (Sigma) was dissolved in the medium and used at 25 μM final concentration. The drugs were maintained during OGD and for 24 h after OGD.

Extraction of Nuclear/Cytoplasmic Fractions

The cortices from HIF1α WT and KO mice were dissected 24 h after the HI procedure, snap-frozen and stored at –80 °C until use. Nuclear and cytoplasmic proteins were extracted with NE-PER™ nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's protocol. Briefly, the tissue was homogenized in 250 μL ice-cold CER I buffer with Halt™ protease and phosphatase inhibitors (Pierce Biotechnology). After incubation with 13.75 μL CER II buffer and vortex, the sample was centrifuged for 10 min at 16,000 *g* at 4 °C and the supernatant was saved as the cytoplasmic extract. The pellet was resuspended in 100 μL ice-cold NER buffer, shaken at 1,500 rpm in the cold room for 20 min, and centrifuged at 16,000 *g* at 4 °C for 30 min. The resultant supernatant was considered as the nuclear extract. The cytoplasmic and nuclear protein aliquots were stored at –80 °C until use. Protein concentrations were measured by BCA assay kit (Pierce). The nuclear and cytosol protein was prepared from primary cortical neurons using the same kit.

Western Blot

For Western blot analysis, equal amounts of protein samples (25–30 μg of cytoplasmic protein or 10–15 μg of nuclear protein) were applied to 4–12% Bis-Tris SDS polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The blots were probed with the following primary antibodies overnight at 4 °C: rabbit anti-HIF1α (Noves Biologicals LLC, Littleton, CO, USA); rabbit anti-Epo (Santa Cruz Biotechnology Inc.; Dallas, TX, USA); rabbit anti-VEGF (Santa Cruz Biotechnology Inc.); rabbit anti-histone 3 (Cell Signaling Technology, Boston, MA, USA); mouse anti-α spectrin (Millipore, Billerica, MA, USA) and β-actin (Santa Cruz Biotechnology Inc.). Horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary IgG antibodies (Santa Cruz Biotechnology Inc.) were used, and the signal was visualized with enhanced chemiluminescence (Amersham, GE Healthcare). Image J software was used to measure the mean optical densities (OD) and the area of protein signal on radiographic film after scanning.

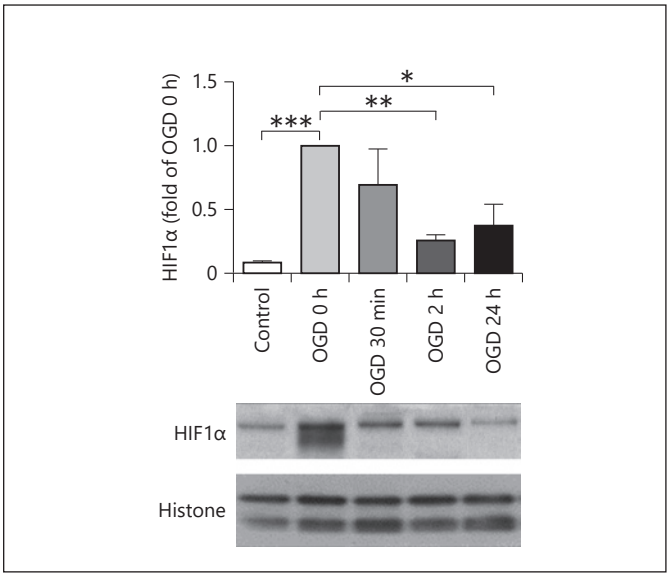

Fig. 1. The time course of HIF1α expression after OGD in primary cortical neurons. HIF1α expression peaked immediately after OGD (0 h) and decreased over time after reoxygenation (* $p < 0.05$, ** $p < 0.01$ vs. OGD 0h; *** $p < 0.001$ vs. control cells; $n = 4–5$).

LDH Assay

This assay was carried out with the primary neurons cultured on the 96-well plates in 100 μL of medium. Neuronal cell death was quantified by a colorimetric cytotoxicity detection kit (Roche, Indianapolis, IN, USA) based on the measurement of lactate dehydrogenase (LDH) activity released from the damaged/dead cells into the culture medium. Fifty microliters of medium were collected immediately after OGD (0 h) from the control or OGD-treated wells to represent cell death during the 6 h of OGD, and again at 24 h after OGD to indicate cell death during 24 h of reoxygenation. At the end of 24 h, the medium was completely changed, and the plates were frozen at –80 °C for at least 2 h and then thawed. Another 50 μL of medium was collected to calculate LDH released from the cells that survived OGD. The percentage of cell death was expressed as: (LDH value at 0 h + at 24 h)/(LDH value at 0 h + at 24 h + after freeze-thaw cycle) × 100.

Statistical Analysis

LDH results and optical density data from Western blot are presented as mean ± SD and were evaluated statistically using the SAS Wilcoxon-Mann-Whitney test. Differences were considered significant at $p < 0.05$.

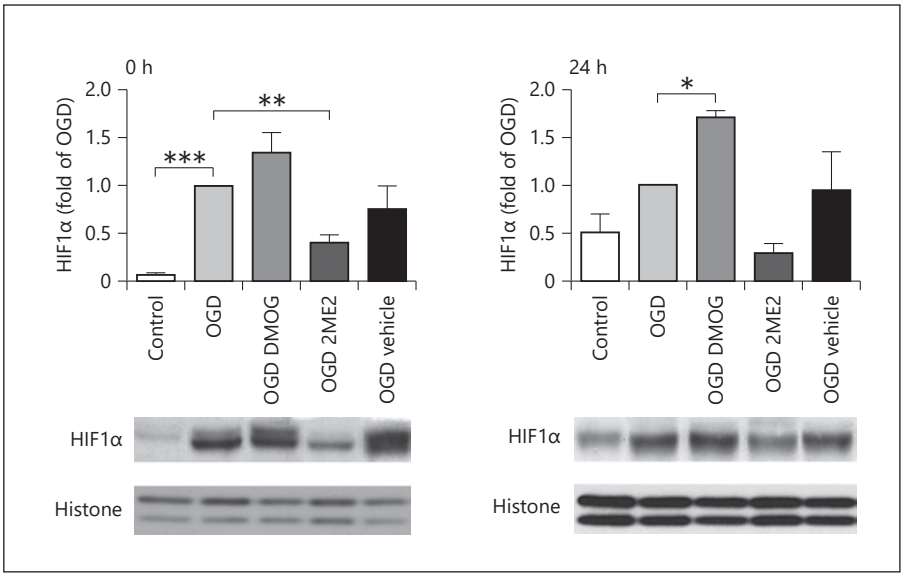

Fig. 2. Manipulation of HIF1α protein by 2ME2 and DMOG 2ME2 inhibited HIF1α expression at 0 h, while DMOG stabilized HIF1α for at least 24 h (* $p < 0.05$, ** $p < 0.01$ vs. OGD; *** $p < 0.001$ vs. control cells; vehicle was 0.5% DMSO. $n = 4–6$).

Results

Pharmacological Manipulation of HIF1α Protein in Primary Cortical Neurons

The expression of HIF1α protein was measured by Western blot at different time points following OGD, an in vitro model of HI. Figure 1 showed that HIF1α accumulated during the 6 h of OGD (measured immediately after OGD, i.e., OGD 0 h) and was degraded upon reoxygenation. HIF1α expression decreased over time up until 24 h. 2ME2, but not the vehicle control (0.5% DMSO), inhibited the increase of HIF1α during OGD, while DMOG stabilized the protein for at least 24 h (Fig. 2). HIF1α was maintained at a lower level at 24 h with 2ME2 treatment, but did not differ significantly from with OGD only (Fig. 2).

HIF1α Inhibition Enhances Necrotic and Apoptotic Cell Death after OGD

To study the role of HIF1α in the endogenous protective responses following OGD, the HIF1α inhibitor 2ME2 or the stabilizer DMOG was added during and after OGD, to determine the effect on cell viability. The LDH analysis (Fig. 3a) showed that 2ME2 (50 μM) or DMOG (25 μM) was not toxic to the neurons under the control normoxic condition. OGD increased cell death from 20.03 ± 2.64% (control) to 41.35 ± 5.76% (OGD) 24 h afterwards ($p < 0.05$ vs. control). HIF1α inhibition further augmented neuronal death (53.57 ± 1.98%, $p < 0.05$ vs. OGD) suggesting that HIF1α is involved in protective mechanisms after OGD. There was no difference following DMOG treatment compared to with OGD alone. Increased spectrin cleavage by calcium-sensitive proteases such as calpain and caspases has been used as an indicator of necrotic and apoptotic cell death. Spectrin cleavage leads to an elevated generation of spectrin breakdown products (SBDPs) of 145/150 kDa (indicative of necrotic and excitotoxic neuronal death) and 120 kDa (represents apoptotic death) [37]. As shown in Figure 3b, OGD induced the production of 145/150-kDa and 120-kDa SBDPs at 0 h and 24 h after OGD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control), which was further increased by HIF1α inhibition with 2ME2 (* $p < 0.05$, ** $p < 0.01$ vs. OGD alone). These data are consistent with the LDH results. In contrast, stabilizing HIF1α with DMOG attenuated the expression of 145/150-kDa SBDPs immediately after OGD, but not 24 h later.

Epo and VEGF Expression following OGD

Epo and VEGF are known HIF1α substrates that are involved in neuroprotection in the rodent model of neonatal HI and neonatal stroke [22, 38]. Under our in vitro condition, VEGF expression was slightly increased 24 h after OGD ($p < 0.05$, OGD vs. control; Fig. 4), while Epo was not induced at these early time points. HIF1α inhibition with 2ME2 diminished the levels of Epo and VEGF immediately after OGD ($p < 0.001$, OGD 2ME2 vs. OGD alone; Fig. 4), but not 24 h later. DMOG, however, increased the expression of VEGF 24 h after OGD ($p < 0.05$, OGD DMOG vs. OGD alone; Fig. 4).

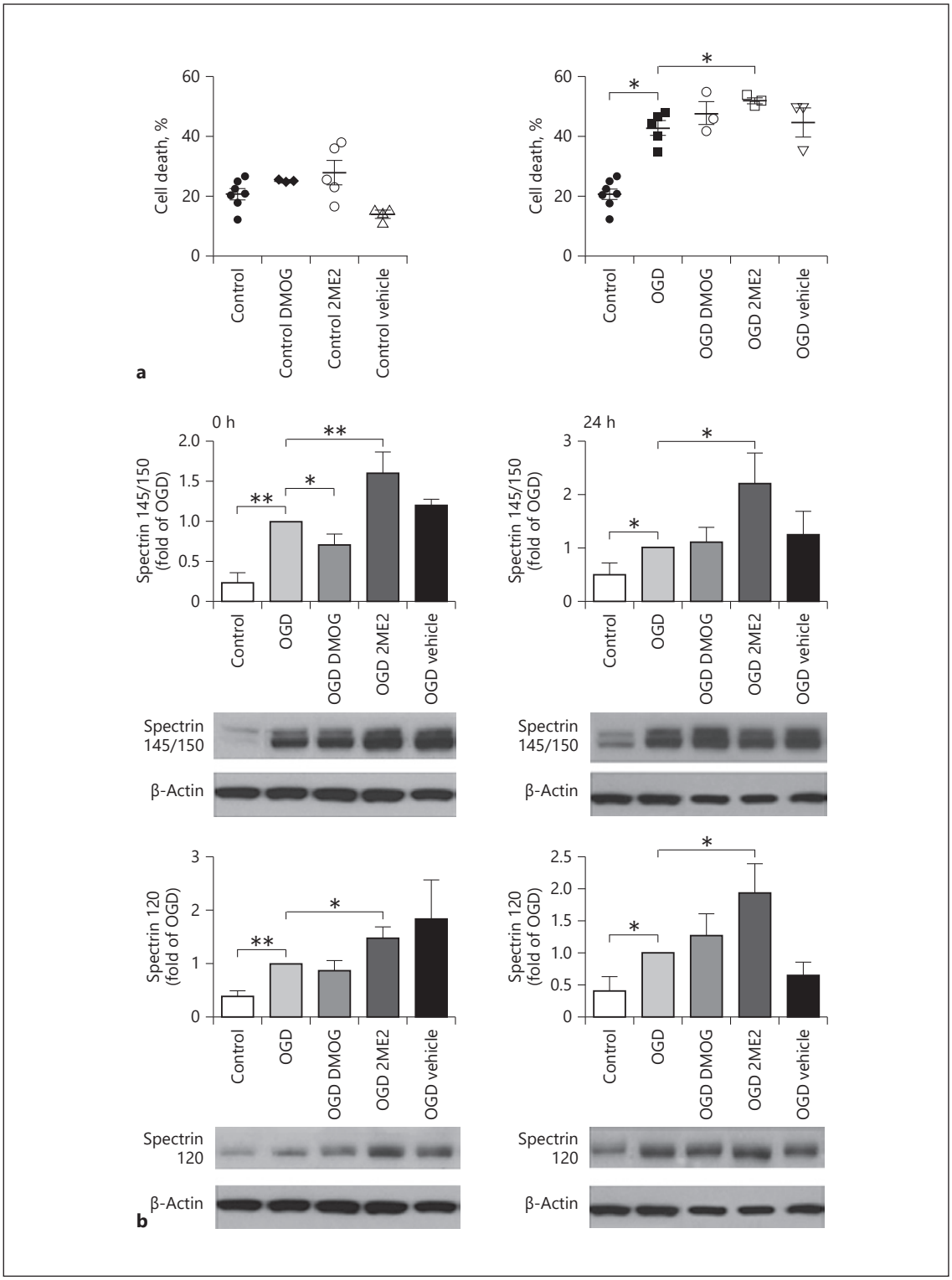

Fig. 3. HIF1α inhibition enhanced necrotic and apoptotic cell death after OGD. **a** 2ME2 exposure during and after OGD caused more cell death than OGD alone by LDH assay (right). The drugs were not toxic to control cells (left). * $p < 0.05$, $n = 5–7$. **b** 2ME2 increased the levels of 145/150-kDa and 120-kDa SBDPs at 0 and 24 h after OGD compared to the OGD alone group. DMOG reduced 145/150-kDa SBDP expression right after OGD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 4–5$).

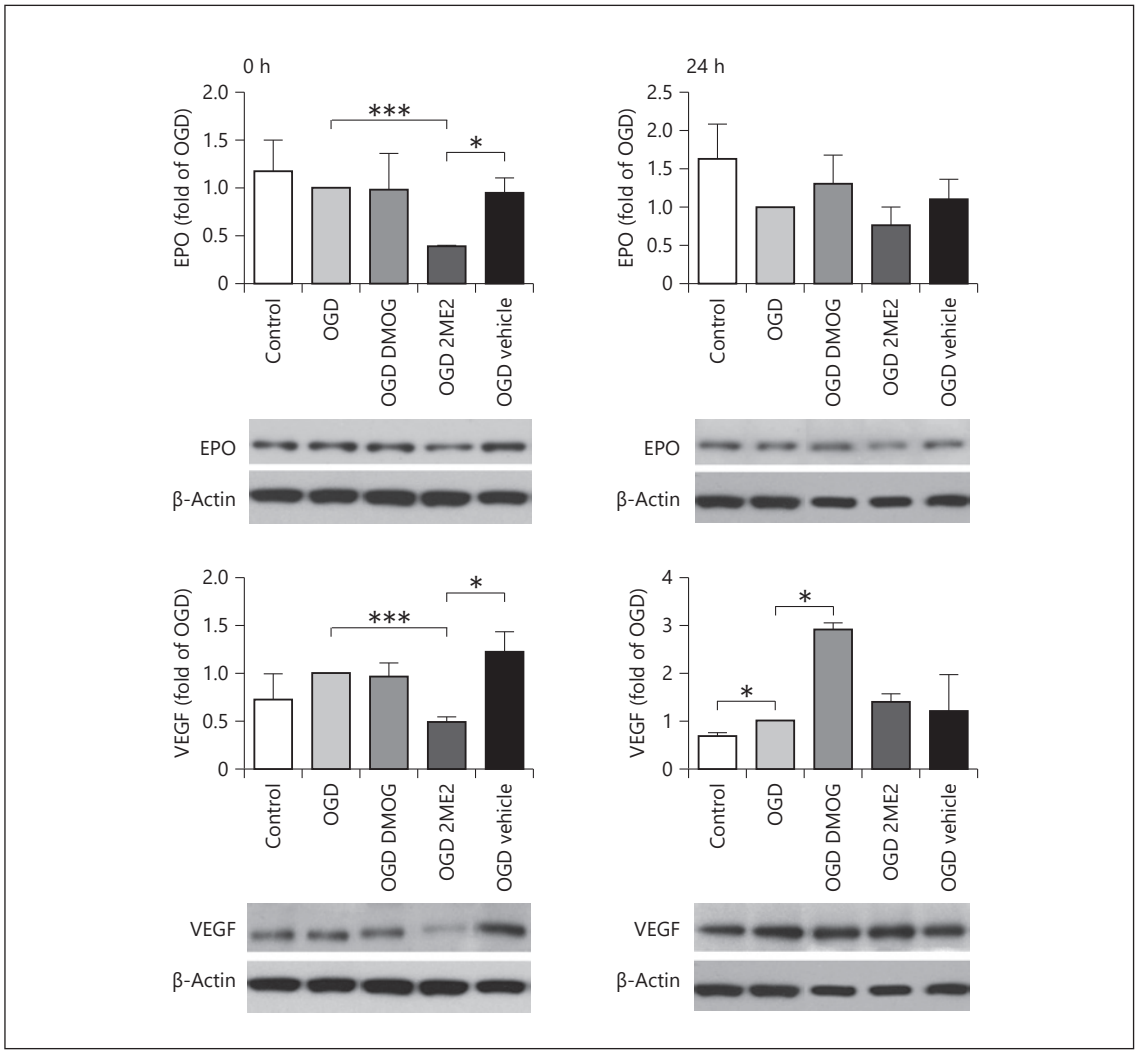

Fig. 4. EPO and VEGF expression following OGD 2ME2 (but not the vehicle control) diminished Epo and VEGF expression at 0 h compared to the OGD-alone group (*** $p < 0.001$). DMOG increased VEGF levels 24 h after OGD (* $p < 0.05$; $n = 3–5$).

Neuronal-Specific HIF1α KO Mice Have More Severe Brain Injury after Neonatal HI

We showed previously that HIF1α-deficient mice have more exacerbated brain injury at P7 than their WT littermates following HI [21]. At the more mature stage of P9, these mice are similarly more vulnerable to HI brain damage. The expression of HIF1α protein was confirmed to be minimal in the HIF1α KO mice 24 h after HI (Fig. 5; * $p < 0.05$, WT sham vs. KO sham, *** $p < 0.001$, WT HI vs. KO HI). Compared to the WT pups, the neuron-specific HIF1α KO mice had significantly higher levels of apoptotic cell death, evaluated by the expression of 120-kDa SBDPs (Fig. 5; * $p < 0.05$, WT HI vs. KO HI). These data agree with our observation when the mice were used at P7 in the same HI model.

Discussion

Using genetic and acute pharmacological manipulation of HIF1α, we examined the function of neuronal HIF1α and its key effectors that may protect the neonatal brain or immature neurons against HI injury in vivo and in vitro. We demonstrated that the inhibition of HIF1α

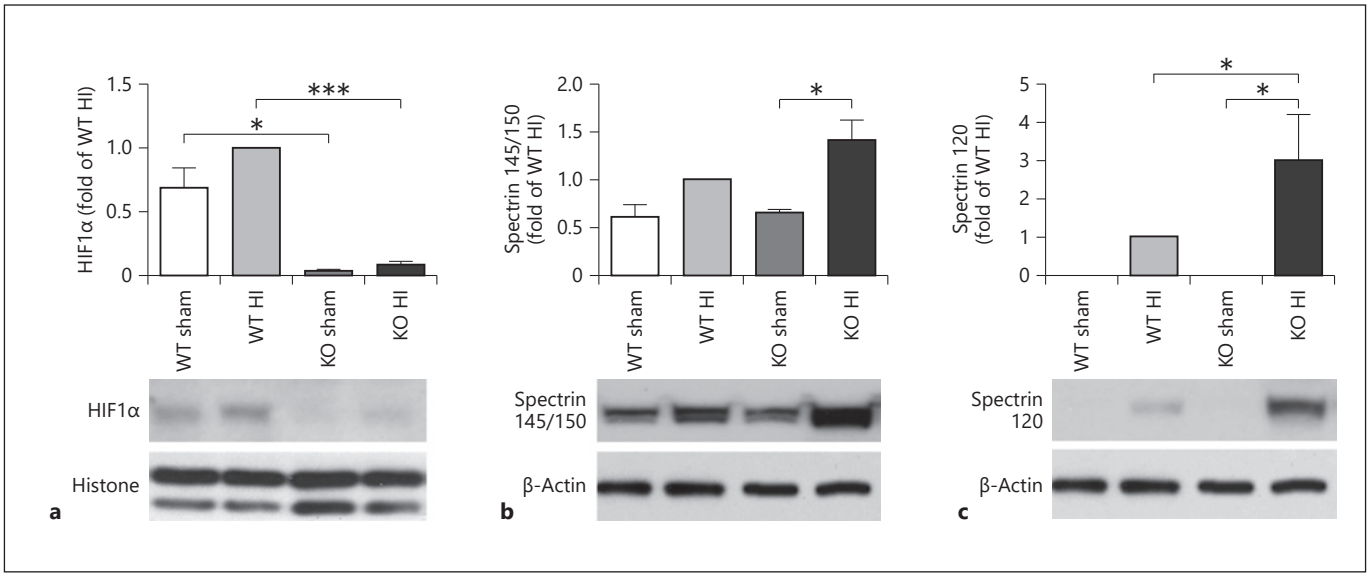

Fig. 5. Neuron-specific HIF1α KO mice had more severe brain injury than WT mice at P9 after HI. **a** HIF1α expression was abolished in the KO sham and HI-injured animals. The levels of 145/150-kDa SBDPs (**b**) and 120-kDa SBDPs (**c**) were significantly higher in the KO mice than in the WT animals at 24 h post-HI. (* $p < 0.05$, *** $p < 0.001$; $n = 4–7$).

with 2ME2 in cortical neurons or the deletion of neuronal HIF1α in P9 mice increased both necrotic and apoptotic cell death following HI, confirming the role of neuronal HIF1α signaling in HI neuroprotection in the developing brain. The in vivo findings are in agreement with our previous data using the same HIF1α-deficient mice at P7 [21].

2ME2 is an endogenous metabolite of estradiol and is the most commonly employed HIF1α inhibitor. The mechanism for its inhibition on HIF1α in a human tumor cell line and in human umbilical vein endothelial cells is that 2ME2 inhibits HIF1α mRNA translation/de novo synthesis, and its association with microtubules, thereby blocking HIF1 nuclear accumulation and its transcriptional activity [39–41]. In the adult rodent brain, administration of 2ME2 before or soon after insult protected against global ischemia [42] or middle cerebral artery occlusion (MCAO)-induced brain damage [43, 44], although 1 study reported that 2ME2 given 10 min after ischemia exacerbated apoptotic death in the CA1 region of the hippocampus following global ischemia [45]. 2ME2 treatment at later time points (8 h after MCAO) was ineffective or even detrimental [46]. This was also true in neonatal HI where 2ME2 showed protection by reducing brain edema and infarct volume when used 5 min after HI, whereas the protection was lost if it was administered 3 h after HI [47, 48]. It is believed that HIF1α induction at an early stage activates the prodeath genes involved in apoptosis and autophagy, while late-stage HIF1α increases substrates, including VEGF, that improve functional recovery and brain repair [28, 46].

In our primary cortical neuronal cultures, 2ME2 effectively inhibited HIF1α accumulation during the 6 h of OGD. As a consequence, neurons with 2ME2 treatment had significantly more cell death, evaluated by 2 different cell viability assays (LDH measurement and SBDP levels). The increased neuronal death was not due to 2ME2 toxicity because, at the 50 μM dose, it did not cause additional death under normoxia when incubated for 30 h. On the other hand, when HIF1α was stabilized with the PHD inhibitor DMOG, while the LDH assay did not show any changes in cell death, it reduced the levels of 145/150-kDa SBDPs right after OGD. DMOG was used at 25 μM since the higher concentrations (it was tested at 50, 100, and 150 μM) were toxic to primary neurons.

These pharmacological data support the protective effects of HIF1α in immature neurons, while in the in vivo models 2ME2 may regulate HIF1α in glial cells or other cell populations that collectively modify the overall pattern and severity of cell death. Furthermore, although 2ME2 is relatively specific for HIF1α inhibition, DMOG, as a competitive inhibitor of PHD used for HIF1α stabi-

lization, also increases HIF2α expression. DMOG is a synthetic analog of α-ketoglutarate and a dimethyl ester of N-oxalylglycine, and therefore has multiple pharmacological effects including the direct inhibition of mitochondrial function that precedes HIF target gene expression [49]. A more specific strategy for an HIF1α gain-of-function study is needed to further clarify its role in neonatal brain HI.

The expression of VEGF and Epo was measured in the primary neurons to identify the HIF1α-mediated protective signaling pathways. These are important HIF1α target substrates implicated in angiogenesis and neurogenesis, respectively, and have therefore been a focus of investigation for promoting recovery and regenerative repair after brain ischemia in rodents and humans [50–53]. Under culture conditions, VEGF was upregulated 24 h after OGD, suggesting that it could be a downstream event following HIF1α activation, while Epo expression remained unchanged in the first 24 h after the insult. In a rat neonatal stroke model, we demonstrated that HIF1α and VEGF are colocalized in neurons with a similar time course of expression [28]. Epo is increased early in neurons, but later in astrocytes [29].

Recent studies indicate that HIF2α has emerged as the transcription factor that regulates Epo synthesis in the kidney, liver, and brain [54–56]. Epo transcription and secretion are increased by hypoxia more in cortical astrocytes where HIF2α is located than those in the neurons [57]. This may explain why we did not observe changes in Epo expression in our primary neurons, or alternatively, Epo may have been induced after 24 h in our in vitro system. It is not clear whether Epo expression in different cell types or at different time points has distinct significance, but we found that exogenous Epo either given early or delayed after transient neonatal stroke improves histological and behavioral outcomes in P10 rats [38, 58]. Interestingly, despite VEGF and Epo not being enhanced right after 6 h of OGD, 2ME2 reduced their levels at this early time point, in parallel with its inhibition of HIF1α expression. In contrast, overexpression of HIF1α by DMOG at 24 h after OGD was accompanied by an increase in VEGF at the same time. The correlated timing and pattern of the changes suggest that VEGF and perhaps Epo are the possible HIF1α downstream mediators for neuroprotection. However, we cannot make this conclusion without inhibition of VEGF or Epo signaling. In our transient neonatal stroke model, treatment with SU5416, a VEGF receptor antagonist, enhanced cell death and limited angiogenesis [59], implying that VEGF plays a role in recovery and repair in brain ischemia. VEGF is also involved in subventricular zone remodeling following neonatal HI, where VEGF-A and VEGF-C are produced by glial progenitor cells and astrocytes. VEGF-A increases astrocyte proliferation and contributes to astrogliogenesis, whereas VEGF-C enhances the proliferation of oligodendrocyte progenitors thereby promoting myelination and white matter regeneration after the injury [60].

The results from neuron-specific HIF1α KO mice are in agreement with our in vitro data on HIF1α inhibition in primary neurons. They also confirm our previous findings that these mice have, overall, increased brain injury at P7 after neonatal HI [21], suggesting that the HIF1α responses are similar in this range (P7–P9) of the developmental stage. The KO mice showed a significantly higher level of 120-kDa spectrin than the WT pups after HI, but not as much 145/150-kDa spectrin, indicating that HIF1α pathways might protect the cells against apoptosis, rather than the necrotic cell death in our neonatal HI model.

Taken together, we demonstrated, in vitro and in vivo, that HIF1α signaling mediates the endogenous protective responses after neonatal brain HI. It plays a key role in promoting neuronal survival by upregulating its target protective or repair genes, including VEGF and Epo, or possibly via other molecular pathways. Identifying the proteins involved in the stabilization and activation of HIF1α could be advantageous in treating neonatal brain ischemic conditions and aid in the discovery of new therapeutic approaches to neonatal HI.

Acknowledgements

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Statement of Ethics

All animal experiments were approved by the University of California San Francisco (UCSF) institutional animal care and use committee.

Disclosure Statement

The authors declare they have no conflicts to disclose.

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