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Divergent effects of estradiol and the estrogen receptor-alpha agonist PPT on eating and activation of PVN CRH neurons in ovariectomized rats and mice

Thammacharoen, S; Geary, N; Lutz, T A; Ogawa, S; Asarian, L
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Abstract

Eating is modulated by estradiol in females of many species and in women. To further investigate the estrogen receptor mechanism mediating this effect, ovariectomized rats and mice were treated with estradiol benzoate or the estrogen receptor-alpha (ER-alpha)-selective agonist PPT. PPT inhibited eating in rats much more rapidly than estradiol (approximately 2-6 h versus >24 h). In contrast, the latencies to vaginal estrus after PPT and estradiol were similar (>24 h). PPT also inhibited eating within a few hours in wild-type mice, but failed to inhibit eating in transgenic mice deficient in ER-alpha (ERalphaKO mice). PPT, but not estradiol, induced the expression of c-Fos in corticotrophin-releasing hormone (CRH)-expressing cells of the paraventricular nucleus (PVN) of the hypothalamus within 90-180 min in rats. Both PPT and estradiol reduced c-Fos expression in an ER-alpha-containing area of the nucleus of the solitary tract. The anomalously rapid eating-inhibitory effect of PPT suggests that PPT's neuropharmacological effect differs from estradiol's, perhaps because PPT differentially activates membrane versus nuclear ER-alpha or because PPT activates non-ER-alpha membrane estrogen receptors in addition to ER-alpha. The failure of PPT to inhibit eating in ERalphaKO mice, however, indicates that ER-alpha is necessary for PPT's eating-inhibitory action and that any PPT-induced activation of non-ER-alpha estrogen receptors is not sufficient to inhibit eating. Finally, the rapid induction of c-Fos in CRH-expressing cells in the PVN by PPT suggests that PPT elicits a neural response that is similar to that elicited by stress or aversive emotional stimuli.
Review article

The role of hypoxia-inducible transcription factors in the hypoxic neonatal brain

Regina Trollmann a,*, Max Gassmann b

a Department of Pediatrics, Friedrich-Alexander-University of Erlangen-Nuremberg Loschgestrasse 15, 91054 Erlangen, Germany
b Institute of Veterinary Physiology, and Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Switzerland

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Abstract

Hypoxia-inducible transcription factors (HIF)-1 and HIF-2, composed of an oxygen-dependent α-subunit and a constitutive β-subunit, have been characterized as the most important regulators of oxygen homeostasis during physiological and pathological conditions. During embryonic, fetal and postnatal brain development, HIFs and specific HIF target genes are involved in early and highly active maturational processes by modulating cell differentiation, vascular development, angiogenesis and metabolic homeostasis. Under hypoxic conditions, activation of the HIF system reflects an immediate and cell-specific response to acute brain hypoxia. In a complementary fashion, both HIF-1 and HIF-2 modulate cerebral hypoxic stress responses and activate endogenous neuroprotective systems during acute and late stages of hypoxic/ischemic (HI) damage of the developing brain. Therefore, HIFs and their specific target genes that are expressed during brain injury are of particular interest for future diagnostic and therapeutic options in HI injury of the developing nervous system.

Keywords: Developing brain; Neuroprotection; VEGF; Mouse placenta; Hypoxic-ischemic encephalopathy

1. Introduction

Hypoxic and ischemic complications during the pre- and perinatal period are common causes of acquired neonatal brain damage associated with severe long-term neurodevelopmental disabilities. The broad spectrum of risk factors includes acute hypoxic/ischemic (HI) injury at birth arising from impaired materno-/feto-placental blood flow or acute anemia, as well as from chronically compromised prenatal fetal oxygen and energy supply, e.g. due to placental abnormalities or maternal diseases [1]. Resulting patterns of HI brain injury are periventricular lesions in the preterm newborn, and cortico-subcortical lesions, especially in the senso-motor cortex and the parasagittal region, and deep gray matter lesions of basal ganglia and thalamus in the near-term and term newborn [2]. This selective vulnerability of the brain to HI is mainly dictated by the stage of brain maturation and severity of hypoxia [3]. Inflammatory (e.g. IL-6, IL-1β, TNF-α), excitotoxic (e.g. glutamate, NMDA-R, AMPA-R) and apoptotic pathways are involved in the complex neurotoxic cascade following HI brain injury [3] and activate a process of self-sustaining secondary neurodegeneration in vulnerable CNS regions [4]. In addition, the availability of endogenous adaptive mechanisms modifying early and delayed stages of hypoxia-induced molecular cascade has been proposed as a crucial factor in the pathophysiology of HI damage of the developing brain. Among these adaptive systems, hypoxia-inducible transcription factors (HIFs) are of particular interest because of (a) their crucial adaptive role during immediate cerebral response to HI, and (b)
their ability to induce vasoactive and metabolic cytoprotective mechanisms during late stages of HI damage of the developing brain. These observations implicate HIFs as diagnostic and therapeutic options in HI injury of the developing nervous system.

2. Hypoxia-inducible transcription factors

Hypoxia-inducible transcription factors (HIF)-1 and HIF-2 have been characterized as the most important regulators of O2-dependent gene transcription modulating oxygen and metabolic supply during hypoxia. HIFs are heterodimers of HIF-α (isoforms HIF-1α, HIF-2α, HIF-3α) and HIF-β (also termed ARNT, aryl hydrocarbon receptor nuclear translocator) subunits that all belong to the PAS family of basic helix-loop-helix (bHLH) transcription factors. Under normoxic conditions, HIF-α subunit is rapidly degraded by the ubiquitin-proteasome pathway mediated by specific prolyl residues. These residues are hydroxylated by HIF-prolyl hydroxylases (prolyl hydroxylation domain protein [PHD]), a process requiring di-oxygen and 2-oxoglutarate as co-substrates. Reduced activity of the PHDs under hypoxia initiates stabilization of the HIF-α subunit, heterodimerization with the β-subunit and activation of nuclear translocation that is followed by binding of the HIF heterodimer to hypoxia response elements of enhancers and promoters of specific target genes (Fig. 1). As a result, numerous HIF target genes modify oxygen and energy supply, e.g. by activation of glucose utilization (e.g. GLUT-1), vasoproliferative and vasoactive effects (e.g. vascular endothelial growth factor, VEGF; inducible NO synthase, iNOS) and cell survival (e.g. erythropoietin, EPO; insulin-like growth factor-1, IGF-1) (for review, see [5]). Interestingly, specific hypoxia response elements have been identified in the promoter region of HIF prolyl hydroxylases PHD2 and PHD3 inducing an autoregulatory feedback control that may prevent overstimulation of the HIF system during persisting hypoxia and reoxygenation [6]. The most widely expressed α-subunit is HIF-1α that was originally identified by affinity purification using oligonucleotides from the EPO locus [7]. As shown by knock-out studies [8], HIF-1α and HIF-2α (also known as

![Diagram of HIF-1 stabilization and activity under normoxia and hypoxia.](image)

Fig. 1. HIF-1 stabilization and activity under normoxia and hypoxia. Under normoxic conditions, hydroxylation at specific proline residues leads to binding of HIF-1α to VHL followed by HIF-1α destruction via the ubiquitin/proteasome pathway. During hypoxia, HIF-1α subunit is stabilized and dimerizes with the ubiquitously expressed HIF-1β subunit. Activation of nuclear translocation is initiated followed by binding of the HIF-1 heterodimer to hypoxia response elements (HRE) of enhancers and promoters of specific target genes. OH, hydroxyl group; VHL, von Hippel-Lindau tumor suppressor protein; P, phosphorylated subunit.

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EPAS1, HOP2, HLF) have unique rather than redundant functions. Complementary action of the two α-subunits in the brain is further assumed e.g. by cell-type specific regulation and differences in target gene specificity [9–11]. For example, transcription of EPO during cerebral hypoxia is mainly regulated by HIF-2α, but not HIF-1α [9]. In contrast, transcriptional activity of genes encoding for enzymes of glycolytic pathway is under control of HIF-1α rather than HIF-2α [12].

Beside hypoxia, nitric oxide, metal ions (e.g. cobalt, nickel) and iron chelators (e.g. desferrioxamine) induce HIF-1α protein stabilization and transcriptional activity at the level of PHDs even under normoxic conditions [5]. Carbon monoxide induces tissue hypoxogenation by diminishing O₂-carrying capacity inhibiting HIF-α protein degradation in vivo as shown in adult rodent brain [11,13].

3. Role of HIFs during early brain development

During embryo- and organogenesis including brain development physiological hypoxia stimulates vascular development, angiogenesis and metabolic adaptation controlled by the HIF regulatory cascade [14,15]. During normal mouse brain development, hypoxic regions were detected from ED 8.5 to 9.0 in neuronal mesenchymal tissue associated with HIF-1α and VEGF co-localization [14]. HIF-1α protein detectable in normoxic developing mouse brain at GD 20 [16] and P7 [17] reflects persisting availability of HIF-1α in the regulation of physiological oxygen demands during neonatal period thereby ensuring fast and adequate adaptation to physiological fluctuations of cellular oxygen tension. Moreover, the crucial role of HIFs and HIF target genes for physiological vasculo- and angiogenesis and brain development is emphasized by knockout experiments. For example, HIF-1α knockout mouse embryos exhibit defects in neural development such as open neural tubes and abnormal cephalic vascularisation and lethality at ED 8.5 [8]. Embryonic brain defects in EPO and EPO-R null mice (lethality at around ED 13.5) consist of a thinning of the neuroepithelium, small brain size and incomplete closure of the neural tube [18].

4. Activation of the HIF system in hypoxic developing brain

Different experimental neonatal rodent models of hypoxic preconditioning [19,20], focal hypoxic/ischemic [19,21] and global non-ischemic hypoxic brain injury [16,17] demonstrated up-regulation of cerebral HIF proteins indicating their role in hypoxic stress-induced molecular responses of the developing nervous system. By establishing a mouse model of late-gestation intrauterine hypoxia in mice our group investigated endogenous HIF regulation during acute hypoxia (6% O₂ for 6 h) in the very immature mouse brain at the last day of mouse gestation (GD 20) [16] that approximately corresponds to the stage of brain maturation of a preterm newborn at mid-gestation [22]. Regional and cell-specific HIF-1α and HIF-2α protein accumulation due to hypoxia in developing mouse brain (Fig. 2) reflects immediate cerebral hypoxic stress responses in selectively vulnerable regions to hypoxia such as cerebral cortex, hippocampus and subventricular zone [4]. Cell-type specific regulation of HIF responses to brain hypoxia is evident from in vitro and in vivo studies, however, in vivo HIF response to hypoxia of the developing brain has not being extensively studied. In adult rodent brain, hypoxia induces stabilization and activity of HIF-1α protein in neurons, astrocytes, ependymal cells and endothelial cells [9,10,23], and HIF-2α protein in astrocytes, endothelial cells and Purkinje cells of the cerebellum [9,11]. Our studies performed at very early stage of mouse brain maturation (GD 20; Fig. 2) showed that HIF-1α is most prominently accumulated in cortical neurons and, to a lower extent, in glial cells and vascular endothelial cells of hippocampus and periventricular zone upon exposure to systemic hypoxia (6% O₂, 6 h). In contrast to a report on P6 rat brains undergoing hypoxic preconditioning (8% O₂; 3 h) [20], we showed cerebral HIF-2α induction upon exposure to hypoxia in the immature mouse brains. This data suggest a differential sensitivity of the HIF system that is attributable to developmental stage, duration and severity of hypoxia as well as cell type [9,12,20]. This is also the case for HIF target gene activation. At early stage of brain development (GD 20), especially VEGF seems to belong to the group of HIF target genes that are activated during early hypoxic response of the developing brain in contrast to EPO, GLUT-1 and iNOS [16,17]. Neuroprotective effects of VEGF have been shown in vitro and in vivo in postnatal rodent brain in response to ischemia and hypoxia [24,25]. VEGF is characterized as a neuronal survival factor by mediating both auto- and paracrine signaling functions in neurons in vitro [25] via its Flk-1 receptor and phosphorylation of downstream signaling molecules including MAPK, p90RSK, and STAT family members. Astrocyte-derived VEGF mediates survival and tube stabilization of hypoxic brain microvascular endothelial cells in vitro [26]. Thus, immediate induction of VEGF in developing hypoxic brain might contribute to hypoxic tolerance of neurons and astrocytes.

As to cell-specific target gene expression, in vitro hypoxia (8% O₂, 3 h) prominently induced e.g. VEGF, prolyl-4-hydroxylase α and 12-lipoxygenase in primary astrocytes rather than neurons, whereas adrenomedullin was strongly up-regulated in neurons but not astrocytes [20]. Of note, regulatory network mediating cerebral
oxygen homeostasis involves a variety of transcription factors others than HIFs, such as NF-κB, EGF, insulin signaling pathways and transcriptional regulators Myc, Jun, and p53, modifying glucose metabolism, angiogenic growth factors, cytokines as well as pro-apoptotic factors [12].

5. HIFs as early markers of developing brain hypoxia

Prognostic markers which reliably identify neonates for cerebral HI injury during the first postnatal hours, when neuroprotective therapies might be efficient, are limited [27]. As response of HIFs to changes in cellular

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and 2). In response to acute hypoxia, HIF-1 (B). Of note, analysis of a hypoxic adult brain (A, lane 1) showed a less subunits were detectable in normoxic brains (A and B) and placentas (B). Of note, analysis of a hypoxic adult brain (A, lane 1) showed a less intense HIF-1α band than a normoxic fetal brain (A, compare lanes 1 and 2). In response to acute hypoxia, HIF-1α and HIF-2α protein stabilization is strongly induced in both developing brain and placenta. Adapted from Trollmann et al. [16].

Fig. 3. Western blot analysis of HIF-1α and HIF-2α protein in developing mouse brain and placenta upon exposure of the pregnant mother to normoxia (N) or hypoxia (H; 6% O₂, 6 h) at GD 20. Both α-subunits were detectable in normoxic brains (A and B) and placentas (B). Of note, analysis of a hypoxic adult brain (A, lane 1) showed a less intense HIF-1α band than a normoxic fetal brain (A, compare lanes 1 and 2). In response to acute hypoxia, HIF-1α and HIF-2α protein stabilization is strongly induced in both developing brain and placenta. Adapted from Trollmann et al. [16].

Oxygen tension is immediate, these factors represent promising markers to signal relevant tissue hypoxia. Interestingly, human in vivo studies suggested adrenomedullin and VEGF as early indicators of perinatal brain injury in preterms [28] and term asphyxiated newborns [29,30]. Moreover, several HIF-regulated growth factors measured in cord blood and placenta have been identified to indicate acute or chronic perinatal hypoxia in term newborns [31,32]. Based on existing cell biological data elucidating molecular pathways involved in the immediate responses of the developing brain to hypoxia per se, and considering our mouse model of acute antepartum systemic hypoxia [16] we propose that HIFs and specific target genes represent indicators of cerebral hypoxic distress during acute hypoxia. Specifically, systemic hypoxia at GD 20 induced a simultaneous up-regulation of HIF-1α and HIF-2α in developing mouse brain and placenta implying that placental accumulation of HIFs is indicative for fetal cerebral hypoxic distress (Fig. 3). Of note, acute hypoxia differentially affected transcriptional activity of specific HIF target genes in placenta and brain indicating time- and cell-specific sensitivity of HIF target genes to hypoxia [16] that might have future diagnostic implications. Interestingly, experimental data supported previous observations of increased VEGF expression in human placentas of asphyxiated newborns developing severe hypoxic–ischemic encephalopathy compared to controls [30].

6. HIFs and neuroprotective strategies

Bergeron et al. [23] described protection of adult rodent brain against experimental stroke by “hypoxic preconditioning” which is defined as exposure of rodents to systemic non-lethal hypoxia (8% O₂ for 3 h) before the ischemic event. This “ischemic tolerance” similarly observed due to preconditioning by desferrioxamine and cobalt chloride in adult brain [23] and retina [33] as well as in neonatal rodent brain [19] has been proposed to be the result of HIF-1 and HIF target gene activation (e.g. VEGF, EPO, adrenomedullin). Among these protective target genes, especially EPO has been extensively investigated in terms of its therapeutic use to reduce stroke volume and improve functional outcome after neonatal stroke in rodents. Neuroprotection by recombinant EPO (rEPO) treatment includes many pathways such as diminishing apoptosis by activation of anti-apoptotic proteins bcl-2 and bcl-XL and inhibition of pro-apoptotic caspases, inflammation, excitotoxicity and activation of brain derived neurotrophic factor [34,35]. Moreover, processes of late brain recovery after HI injury are modulated by EPO such as neurogenesis, angiogenesis and migration of regenerating neurons (for review, see [36]). Protective effects have even been found if high-dose rEPO treatment was initiated after the onset of HI injury in neonatal rodents [34]. Human data on neuroprotective effects of rEPO in hypoxic neonatal brain injury is limited. Studies initiated for treatment of anemia showed no significant improvement of neurodevelopmental outcome in preterm neonates who received low-dose rEPO [37]. A recent randomized mono-center study on efficacy and safety of high-dose rEPO treatment in premature infants revealed no significant effects on short-term outcome, however, of importance, no significant adverse effects [38]. Neurobiological characteristics of EPO/EPO-R system during development might explain differences of in vitro and in vivo effects such as limited transport across blood–brain-barrier and delayed hypoxia-induced activation of the heterodimeric low affinity EPO-R [36].

The hypothesis arises as whether it is advantageous to target HIFs for neuroprotection as activation of the broad spectrum of HIF target genes might have synergistic protective effects. Indeed, embryonic neuronal cell culture experiments [39] showed that inhibitors of PHD significantly prevented glutamate-induced neuronal death suggesting PHD as promising targets for protection from oxidative death in neurons.

In summary, at early developmental stage of brain maturation, HIFs well characterized as complementary systems to modulate hypoxic stress responses reflect an immediate and cell-specific response to acute brain hypoxia. Based on experimental data, HIFs and HIF target genes are promising candidates for potential future diagnostic and prognostic marker systems in perinatal HI encephalopathy. Addressing both cellular and regional vulnerability of the developing brain pharmacological activation of HIF-regulated mechanisms might reflect targets for future studies on age-appropriate neuroprotection in newborns.
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