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Research report

Differential regulation of angiogenesis in the developing mouse brain in response to exogenous activation of the hypoxia-inducible transcription factor system

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ABSTRACT

Angiogenesis due to hypoxic-ischemic (HI) injury represents a crucial compensatory mechanism of the developing brain that is mainly regulated by hypoxia-inducible transcription factors (HIF). Pharmacological stimulation of HIF is suggested as a neuroprotective option, however, studies of its effects on vascular development are limited. We analyzed the influence of the prolyl-4-hydroxylase inhibitor (PHI), FG-4497, and erythropoietin (rhEPO) on post-hypoxic angiogenesis (angiogenic growth factors, vessel structures) in the developing mouse brain (P7) assessed after a regeneration period of 72 h. Exposure to systemic hypoxia (8% O_2, 6 h) was followed by treatment (i.p.) with rhEPO (2500/5000 IU/kg) at 0, 24 and 48 h or FG-4497 (60/100 mg/kg) compared to controls. In response to FG-4497 treatment cortical and hippocampal vessel area and branching were significantly increased compared to controls. This was associated with elevated ANGPT-2 as well as decreased ANGPT-1 and TIE-2 mRNA levels. In response to rhEPO, mildly increased angiogenesis was associated with elevated ANGPT-2 but also TIE-2 mRNA levels in comparison to controls. In conclusion, present data demonstrate a differential regulation of the angiopoietin/TIE-2 system in response to PHI and rhEPO in the post-hypoxic developing brain pointing to potential functional consequences for vascular regeneration and vessel development.

1. Introduction

Cerebral hypoxia and ischemia remain the most common causes of perinatally acquired brain injury of term and preterm infants. Well-known detrimental consequences are elevated acute mortality and long-term neurodevelopmental morbidity, including behavioral, cognitive and sensomotor deficits as well as refractory epilepsy (Ahearne et al., 2016; Stark et al., 2016). Maturational stage and degree of hypoxia and ischemia are considered the main determining factors modifying the extent and pattern of brain damage as well as dysregulation of crucial maturational processes (Shaikh et al., 2015).

During the complex cytotoxic response to an acute hypoxic-ischemic (HI) insult, the developing brain activates inflammatory and pro-apoptotic pathways over time, resulting in a prolonged period of necrosis and apoptosis in selectively vulnerable regions, primarily the striatum, ventrobasal thalamus and subventricular zone (SVZ) (Chavez-Valdez et al., 2016). During the very early hypoxic period, hypoxia-inducible transcription factors (HIF-1, HIF-2) have been characterized as the most important adaptive modulators of oxygen and energy homeostasis (Semenza, 2014). The oxygen-sensitive HIF-α subunit is rapidly degraded during normoxia by the ubiquitin–proteasome pathway following hydroxylation of specific prolyl residues by HIF prolyl-4-hydroxylases (PHD), requiring iron (Fe^{2+}), di-oxygen and 2-oxoglutarate as co-substrates. During hypoxia, functional activity of these specific PHDs is inhibited, thus, HIF-α protein accumulates with subsequent transcriptional activation of specific target genes.

Abbreviations: CC3, cleaved cysteine-aspartic protease 3; EPO, erythropoietin; HC, hippocampus; HI, hypoxic-ischemic; HIF, hypoxia-inducible transcription factor; PHI, prolyl-4-hydroxylase inhibitor; SVZ, subventricular zone; VEGF, vascular endothelial growth factor; VT, vehicle treated.

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These genes are involved in the compensatory regulation of cell survival, oxygen transport, erythropoiesis (e.g., erythropoietin; EPO), and angiogenesis (vascular endothelial growth factor; VEGF, and its tyrosine kinase receptor VEGFR-1). In addition, HIF-α subunits have emerged as crucial regulators of the VEGF co-receptor neuropilin (NRP)-1, as well as angiopoietin (ANGPT)-1 and the endothelial tyrosine kinase receptor, TIE-2. Among them, ANGPT-1 and TIE-2 are predominantly expressed in endothelial cells and are under the control of HIF-2 (Bain et al., 2013; Park et al., 2016).

Novel low molecular weight HIF prolyl-4-hydroxylase inhibitors (PHI), acting as 2-oxoglutarate analogs, have been proposed as neuroprotective therapeutics for HI (Chen et al., 2014; Ogle et al., 2012; Reischl et al., 2014; Singh et al., 2016; Trollmann et al., 2014) and hemorrhagic brain injury (Karuppagounder et al., 2016). Our previous studies confirmed that the novel PHI FG-4497 is able to increase endogenous expression of neurotrophic factors such as HIF-1, HIF-2, VEGF, adrenomedullin as well as EPO in the normoxic developing mouse brain (Schneider et al., 2009). Among them, anti-apoptotic, anti-oxidative, anti-inflammatory and anti-excitotoxic properties have been comprehensively and specifically demonstrated in response to EPO in neonatal rodent models of inflammatory (Lan et al., 2016) and HI brain injury (Hellewell et al., 2013; Traudt et al., 2013; Van de Looij et al., 2014; Yan et al., 2016; Zhu et al., 2014). EPO activates, via homodimer EPOR binding and Jak2 kinase phosphorylation, multiple signaling pathways, such as MAPK/ERK, PI3K, Stat5 and NF-κB, modifying transcriptional activity of anti-apoptotic factors (e.g., Bcl-2, Bcl-XL). RhEPO plays a vital part in long-term repair through its regenerative potential in neurogenesis, oligodendrogenesis and angiogenesis (Van de Looij et al., 2014; Yan et al., 2016; Zhu et al., 2014) mediated via induction of matrix metalloproteinases (Van de Looij et al., 2014), BDNF/GDNF and VEGF/VEGFR-2 signaling pathways (Yan et al., 2016), as well as attenuation of ERK pathways (Jeong et al., 2017). Clinical studies in preterm and term newborns have confirmed the safety of high-dose rhEPO, as well as improvement of cognitive performance and white matter integrity in preterms (Song et al., 2016) as well as neurodevelopmental outcome and MRI injury volume in term newborns with hypoxic-ischemic encephalopathy (Mulkey et al., 2017; Wu et al., 2016). However, optimal treatment regimens and efficacy in relation to age-specific patterns of neonatal brain injury have yet to be established.

Cerebral post-HI vascular regeneration is mainly regulated by the HIF/VEGF system with basic role of the VEGF co-receptors neuropilins and the angiopoietin/TIE-2 system (Beck et al., 2009; Zhu et al., 2014). ANGPT-1, constitutively expressed in perivascular cells, mostly promotes stabilization, maturation and remodeling of sprouting vascular structures (Nag et al., 2017; Shin et al., 2013), while ANGPT-2, secreted by endothelium-associated pericytes, modulates vascular permeability and microvessel density (Beck et al., 2009; Benderro et al., 2014). However, the precise mechanisms of regulation of the ANGPT/TIE-2 system during development have not yet been elucidated (Yun et al., 2013; Zhou et al., 2016). In terms of vascular regenerative effects it remains to be considered whether the pharmacologic activation of a broad spectrum of HIF targets by PHI differs from treatment effects of one single target (rhEPO). Previously, we showed that FG-4497 protects the developing mouse brain from hypoxia-induced apoptosis (Trollmann et al., 2014). In this study, we further investigated whether there are different effects of PHI (FG-4497) and rhEPO on vascular structures during early postnatal mouse brain development upon acute cerebral hypoxia using the established murine model of perinatal systemic hypoxia. After a regeneration period of three days, cerebral region-specific expression of VEGF as well as expression of the angiopoietin/TIE-2 system were analyzed in relation to hypoxia-, FG-4497- and rhEPO-mediated changes in microvascular structures.

2. Results

2.1. Anti-apoptotic effects of FG-4497 and rhEPO in the hypoxic developing mouse brain

After a 72-h interval of regeneration, we observed a significantly decreased number of TUNEL- (Fig. 1) and CC3-positive cells (Fig. 1_Supplementary Materials) in response to rhEPO in the parietal cortex and HC along with the subventricular zone (SVZ) of hypoxia-exposed brains compared to corresponding areas of vehicle-treated controls. Corresponding data on the anti-apoptotic effects of FG-4497 is published elsewhere (Trollmann et al., 2014).

2.2. Angiogenesis in response to FG-4497 and rhEPO in the hypoxic developing mouse brain

Analysis of cortical and hippocampal vessel development in brains exposed to acute global hypoxia at P7, as assessed by PECAM-1 IHC after the short-term reoxygenation period of 72 h, showed a trend of increased vessel area in the hypoxia-exposed parietal cortex (Figs. 2A and 3A) and HC (Figs. 2 and 3_Supplementary Materials) versus normoxic controls.

FG-4497 led to significantly increased vessel area (Fig. 2A, $P < 0.01$) and branching in the hypoxic parietal cortex (hypoxia-exposed brains, controls (n = 3): 21.4 ± 3.3%, FG-4497 (100 mg/kg, n = 3): 39.1 ± 4.0; $P < 0.01$) compared to controls. Further, FG-4497 treatment also resulted in significantly enlarged vessel area (Fig. 2_Supplementary Materials, $P < 0.05$) and branching (hypoxia-exposed brains, controls (n = 3): 24.0 ± 1.8%, FG-4497 (100 mg/kg, n = 3): 37.3 ± 4.3%; $P < 0.05$) in the developing HC when compared to controls. Representative photomicrographs of PECAM-1 IHC are presented in Fig. 2 (HC: Fig. 2_Supplementary Materials).

In response to rhEPO we also observed significantly higher values for vessel area and branching in the hypoxic parietal cortex and HC (Fig. 3A, $P < 0.05$). The angiogenic effects of rhEPO were quite pronounced also in the normoxic parietal cortex (Fig. 3A, $P < 0.05$). versus controls, there was an increase in vessel area (Fig. 3A, $P < 0.05$), vessel length (controls (n = 3): 34.9 ± 0.9 μm, rhEPO 2500 IU/kg (n = 3): 42.5 ± 2.3 μm; $P < 0.01$) and branching (controls (n = 3): 21.1 ± 1.3%, rhEPO 2500 IU/kg (n = 3): 31.3 ± 2.8 %; $P < 0.05$). In the HC, there was a significant increase of the vessel area in hypoxia-exposed tissues in response to high-dose rhEPO (Fig. 3_Supplementary Materials, $P < 0.05$). Furthermore, vessel branching was rather marked as a result of the treatment in normoxic (controls (n = 3): 19.3 ± 4.1%, rhEPO 2500 IU/kg (n = 3): 34.1 ± 2.0%; $P < 0.05$) as well as hypoxia-exposed tissues (controls (n = 3): 24.0 ± 1.8%, rhEPO 2500 IU/kg (n = 3): 33.6 ± 1.8%; $P < 0.05$). Representative sections of the PECAM-1 IHC are seen in Fig. 3 (HC: Fig. 3_Supplementary Materials).

These vascular effects in response to our treatment regimens were related to specific VEGF mRNA expression analyzed by ISH. While hypoxia mainly increased VEGF mRNA expression in the HC compared to normoxic controls (Fig. 5_Supplementary Materials), FG-4497 (Fig. 4A, $P < 0.05$) as well as rhEpo (Fig. 4B, $P < 0.01$) significantly induced VEGF mRNA expression in the hypoxia-exposed parietal cortex (compared to controls) and in a minor degree in the HC (Fig. 5_Supplementary Materials).

2.3. Differential regulation of angiogenic factors in response to FG-4497 and rhEPO in the hypoxic developing mouse brain

As expected, subsequent to the regeneration period of 72 h, there were no significant differences in HIF-1α and HIF-2α