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REPETITIVE ACUTE INTERMITTENT HYPOXIA INCREASES GROWTH/NEUROTROPHIC FACTOR EXPRESSION IN NON-RESPIRATORY MOTOR NEURONS

I. SATRIOTOMO, [*§] **N. L. NICHOLS,** [†] **E. A. DALE** [‡]
A. T. EMERY J. M. DAHLBERG AND G. S. MITCHELL [§]

Department of Comparative Biosciences, University of Wisconsin, Madison, WI 53706, USA

Abstract—Repetitive acute intermittent hypoxia (rAIH) increases growth/trophic factor expression in respiratory motor neurons, thereby eliciting spinal respiratory motor plasticity and/or neuroprotection. Here we demonstrate that rAIH effects are not unique to respiratory motor neurons, but are also expressed in non-respiratory, spinal alpha motor neurons and upper motor neurons of the motor cortex. In specific, we used immunohistochemistry and immunofluorescence to assess growth/trophic factor protein expression in spinal sections from rats exposed to AIH three times per week for 10 weeks (3 × wAIH). 3 × wAIH increased brain-derived neurotrophic factor (BDNF), its high-affinity receptor, tropomyosin receptor kinase B (TrkB), and phosphorylated TrkB (pTrkB) immunoreactivity in putative alpha motor neurons of spinal cervical 7 (C_7) and lumbar 3 (L_3) segments, as well as in upper motor neurons of the primary motor cortex (M_1). 3 × wAIH also increased immunoreactivity of vascular endothelial growth factor A (VEGFA), the high-affinity VEGFA receptor (VEGFR-2) and an important VEGF gene regulator, hypoxia-inducible factor-1α (HIF-1α). Thus, rAIH effects on growth/trophic factors are characteristic of non-respiratory as well as respiratory motor neurons. rAIH may be a useful tool in the treatment of disorders causing paralysis, such as spinal injury and motor neuron disease, as a pretreatment to enhance motor neuron survival during disease, or as preconditioning for cell-transplant therapies.

Key words: intermittent hypoxia, BDNF, VEGF, TrkB, HIF-1, motor neuron.

INTRODUCTION

System and cellular adaptations to hypoxia are crucial in many physiological and pathophysiological states. At the systems level, intermittent hypoxia (IH) elicits respiratory plasticity, potentially minimizing future recurrence of IH (Mitchell et al., 2001; Feldman et al., 2003; Mitchell and Johnson, 2003; Mahamed and Mitchell, 2007; Devinney et al., 2013). IH-induced plasticity occurs at multiple sites in the neural system controlling breathing, including peripheral chemoreceptors (Prabhakar, 2001, 2011), brainstem integrating neurons (Ling et al., 2001; Kline et al., 2007; Kline, 2010) and respiratory motor nuclei (Baker-Herman and Mitchell, 2002; Baker-Herman et al., 2004).

On a cellular level, IH alters the expression of key molecules associated with both respiratory plasticity and neuroprotection. The most widely studied model of IH-induced respiratory plasticity, phrenic long-term facilitation (pLTF) following acute intermittent hypoxia (AIH), requires spinal serotonin receptor activation (Bach and Mitchell, 1996; Baker-Herman and Mitchell, 2002) and serotonin-dependent synthesis of brain-derived neurotrophic factor (BDNF; Baker-Herman et al., 2004). Repetitive AIH (rAIH) elicits long-lasting increases in the expression of many molecules necessary for pLTF within the phrenic motor nucleus, including BDNF and its high-affinity receptor, tropomyosin receptor kinase B (TrkB) (Wilkerson and Mitchell, 2009; Lovett-Barr et al., 2012; Satriotomo et al., 2012). Apart from its key role in neuroplasticity, BDNF is neuroprotective for neurons stressed by ischemia (Duncan et al., 2004; Ferrer et al., 2004). The transcription factor hypoxia inducible factor 1α (HIF-1α; Semenza, 2007) regulates expression of other growth/trophic factors, such as vascular endothelial growth factor (VEGF) and its high-affinity receptor, VEGFR-2 (Calvani et al., 2012). VEGF and VEGFR-2 are expressed in motor neurons (Yang et al., 2003), elicit respiratory motor plasticity (Dale-Nagle et al., 2011), and are neuroprotective against ischemic injury (van Bruggen et al., 1999; Jin et al., 2000). Thus, BDNF and VEGF are hypoxia-regulated genes that elicit both spinal plasticity and neuroprotection.

*Corresponding author. Address: Department of Physical Therapy, University of Florida, 330 Center Drive, Gainesville, FL 32610, USA. Tel: +1-352-273-5972.
E-mail address: satriotomo@ufl.edu (I. Satriotomo).
[†] Current address: Department of Biomedical Sciences, University of Missouri, Columbia, MO 65211, USA.
[‡] Current address: Department of Integrative Biology and Physiology, UCLA, Los Angeles, CA 90095, USA.
[§] Current address: Department of Physical Therapy and McKnight Brain Institute, University of Florida, Gainesville, FL 32610, USA.
Abbreviations: AIH, acute intermittent hypoxia; BDNF, brain-derived neurotrophic factor; dAIH, daily AIH; HIF-1α, hypoxia-inducible factor-1α; IH, intermittent hypoxia; NTRK2, Neurotrophic Tyrosine Kinase Receptor 2; pLTF, phrenic long-term facilitation; pMF, phrenic motor facilitation; pTrkB, phosphorylated TrkB; rAIH, repetitive acute intermittent hypoxia; TrkB, tropomyosin receptor kinase B; VEGF, vascular endothelial growth factor.

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IH elicits plasticity in neural systems not directly linked to breathing. For example, a single presentation of AIH elicits transient increases in sympathetic nerve activity (Dick et al., 2007; Xing and Pilowsky, 2010) and daily AIH (dAIH) for one week elicits prolonged improvement in forelimb function of rats with cervical spinal injuries, an effect that lasts weeks following treatment (Lovett-Barr et al., 2012; Posser-Loose et al., 2015). A single AIH exposure (15, 1-min hypoxic episodes, 9% inspired O_2; 1-min intervals) improves leg strength in persons with chronic spinal injuries (Trumbower et al., 2012), and dAIH (15, 1.5 min episodes per day, 9% O_2; 1.5 min intervals) and dAIH paired with 30-min of overground walking practice improved walking speed and endurance in patients with chronic incomplete spinal cord injuries (Hayes et al., 2014). Thus, IH may elicit similar plasticity in respiratory and non-respiratory motor systems. Fundamental mechanisms giving rise to such similar functional plasticity have not been adequately explored.

We previously demonstrated that a distinct protocol of repetitive AIH consisting of AIH (10, 5-min episodes of 10.5% O_2 per day; 5-min normoxic intervals) three times per week for 10 weeks (3 × wAIH) elicits neurochemical plasticity in phrenic motor neurons (Satriotomo et al., 2012). Here, we tested the hypothesis that 3 × wAIH also increases the BDNF, TrkB, p-TrkB, HIF-1α, VEGF and VEGFR-2 expression in non-respiratory motor neurons. Immunohistochemical techniques were utilized to localize the expression of these growth/trophic factors and their main receptors in alpha motor neurons innervating upper and lower limbs in C_7 and L_3 ventral gray matter, and in the primary motor cortex (M_1).

An understanding of rAIH-induced growth/trophic factor expression may be useful as we develop therapeutic strategies to treat motor deficits in patients, including those with cervical spinal injuries or motor neuron disease (Dale et al., 2014; Navarrete-Opazo and Mitchell, 2014).

EXPERIMENTAL PROCEDURES

Animals and experimental treatments

Twenty male adult Sprague–Dawley (SD) rats weighing 300–330 g were randomly exposed to normoxia (n = 10) or 3 × wAIH for 10 weeks (n = 10). AIH was accomplished by placing unrestrained rats in Plexiglass chambers (one rat per chamber, dimensions 12 in × 4.5 in × 4.5 in) while gases flushing through the chambers (4 L/min) were alternated between 21% and 10.5% O_2 at 5-min intervals. One day prior to treatment onset, rats were acclimated to the exposure chambers before beginning the 3 × wAIH protocol: 10, 5-min hypoxic episodes (FIO_2 = 0.105), separated by 5-min normoxic intervals (FIO_2 = 0.21), three times per week for 10 weeks as described previously (Satriotomo et al., 2012). Sham rats were in chambers for an equivalent period of time, but did not receive hypoxia. Chamber oxygen levels were continuously monitored (AX300-1, Teledyne Analytical Instruments, City of Industry, CA, USA). Both 3 × wAIH and normoxia-treated rats rested quietly or slept during exposure periods. All procedures in this study were carried out in accordance with the National Institutes of Health (NIH) guidelines for care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at the School of Veterinary Medicine, University of Wisconsin-Madison.

Immunohistochemistry. All rats treated with normoxia or 3 × wAIH were euthanized and perfused transcardially with cold 0.01 M phosphate-buffered saline (PBS, pH 7.4), followed by 4% buffered paraformaldehyde. The brain and spinal cords were immediately removed, and cryoprotected in 30% sucrose at 4 °C until they sank. Transverse sections of the cortical area of the primary motor cortex (M_1), cervical spinal (C_7) and lumbar spinal segments (L_3) were processed for immunohistochemistry. Transverse sections (40 μm) were cut using a freezing microtome (Leica SM 200R, Germany). For immunostaining, free-floating sections were washed in 0.1 M Tris-buffered saline with 0.1% Triton-X100 (TBS-Tx; 3 × 5 min) and incubated (30 min) in TBS containing 1% H_2O_2. After washing (3 × 5 min) in TBS-Tx, tissues were blocked (60 min) with 5% of normal goat serum or normal rabbit serum and then tissue was incubated at 4 °C overnight in primary antibodies: rabbit polyclonal anti-BDNF (N-20, 1/1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit polyclonal anti-TrkB (1/500, Santa Cruz Biotechnology, Santa Cruz, CA); rabbit serum anti phospo-TrkB (1/1000, courtesy of Dr. Moses Chao, NYU); rabbit polyclonal anti-VEGF (A-20, 1/1000, Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal anti-VEGFR-2 or KDR (Kinase insert Domain Receptor) (V3003, 1/500, Sigma–Aldrich, St. Louis, MO, USA) and rabbit polyclonal anti-HIF-1α (1/500, Santa Cruz Biotechnology, Santa Cruz, CA). Following overnight incubation, sections were washed and incubated in either biotinylated secondary goat anti-rabbit antibody (1:1,000, Vector Laboratories, Burlingame, CA, USA) for BDNF, TrkB, phospho-TrkB, and VEGF, or biotinylated secondary goat anti-mouse antibody for VEGFR-2 (1:1000, Vector Laboratories, Burlingame, CA). Conjugation with avidin–biotin complex (Vecstatin Elite ABC kit, Vector Laboratories, Burlingame, CA) was followed by visualization with 3,3′-diaminobenzidine-hydrogen peroxidase (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Sections were then washed in TBS, placed in gelatin-coated slides, dried, dehydrated in a graded alcohol series, and then cleared with xylenes and mounted with Eukitt mounting medium (Electron microscope sciences, Hatfield, PA, USA).

All images were captured and analyzed with a digital camera (SPOT II; Diagnostic Instruments, Sterling Heights, MI, USA). Final photomicrographs were created with Adobe Photoshop software (Adobe System, San Jose, CA, USA). All images received equivalent adjustments to tone scale, gamma and sharpness. Sections incubated without primary or secondary antibodies served as negative controls. In addition we pre-absorbed the primary BDNF and VEGF antibodies with a fivefold (by concentration) excess of specific blocking peptides (sc-546 P and sc-152 P; both from

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