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INTERMITTENT HYPOXIA REGULATES RNA POLYMERASE II IN HIPPOCAMPUS AND PREFRONTAL CORTEX

M. L. IGNACAK,[a] S. V. HARBAUGH,[a] E. DAYYAT,[b] B. W. ROW,[b] D. GOZAL[b] AND M. F. CZYZYK-KRZESKA[a]*

[a]*Department of Cancer and Cell Biology, University of Cincinnati, Cincinnati, OH 45267-0505, USA*

[b]*Kosair Children's Hospital Research Institute, and Departments of Pediatrics, Pharmacology and Toxicology, University of Louisville, Louisville, KY, USA*

Abstract—Intermittent hypoxia (IH) is a major pathological factor in the development of neural deficits associated with sleep-disordered breathing. Here we demonstrate that IH lasting 2 or 30 days, but not sustained hypoxia (SH) of the same duration, was accompanied by several posttranslational modifications of the large subunit of RNA polymerase II, Rpb1, including hydroxylation of proline 1465, phosphorylation of serine 5 residues within the C-terminal domain, and nondegradative ubiquitylation. These modifications were found to occur in two regions of the brain, hippocampal region CA1 and the prefrontal cortex, but not in neocortex, brainstem and CA3 region of hippocampus. We also found that mice exposed to 14 or 30 days of IH, but not SH, demonstrated cognitive deficits in behavioral assays. Furthermore, by using the pheochromocytoma-derived PC12 cell line, we showed that, under *in vitro* IH conditions, induction of Rpb1 hydroxylation, phosphorylation, and ubiquitylation required that the von Hippel-Lindau protein be present. We hypothesize that the observed modifications of Rpb1 participate in regulating the expression of genes involved in mediating cognitive deficits evoked by chronic IH.

Key words: intermittent hypoxia, hippocampus, prolyl hydroxylase, RNA polymerase II, PC12 cells.

Intermittent hypoxia (IH), or repeated episodes of hypoxia followed by re-oxygenation, is associated with many human diseases and is among the prototypic consequences of sleep-disordered breathing (SDB). SDB occurs in 2% to 3% of all children (Lumeng and Chervin, 2008), and in 5% of the middle-aged, and 20% to 30% of the elderly population (Punjabi, 2008). IH has been linked to numerous morbidities in SDB, such as disorders of the Central Nervous System, i.e. decreased cognitive performance, depression, increased prevalence and severity of stroke, attention deficits, and excessive sleepiness (Teran-Santos et al., 1999; Beebe and Gozal, 2002; O'Brien et al., 2003a,b; Morrell and Twigg, 2006; Minoguchi et al., 2007; Bassetti et al., 2006).

Cellular and biochemical changes in the human hippocampus are closely correlated with SDB (Macey et al., 2002; Morrell et al., 2003; Bartlett et al., 2004). Accordingly, our laboratories demonstrated that IH induced oxidative stress (Xu et al., 2004), apoptosis (Gozal et al., 2001a), and specific changes in the protein profile of the hippocampal CA1 area (Gozal et al., 2002; Klein et al., 2002), which coincided with an impaired acquisition and retention of cognitive spatial tasks, as demonstrated by the Morris water maze (Gozal et al., 2001a,b, 2003; Row et al., 2002, 2003). We also showed that IH substantially decreased phosphorylation of CREB Ser-133, without effects on the total expression of CREB, specifically within the CA1, and not the CA3, region (Goldbart et al., 2003). In addition, IH decreases the resting potential, causes partial depolarization and diminished sodium currents (Gu and Haddad, 2001), changes enzyme activity (Marzatico et al., 1986), and impairs the ability of CA1 neurons to induce and maintain population-spike long-term potentiation (Payne et al., 2004). However, the molecular and cellular mechanisms by which IH induces oxidative stress, causes neuronal loss of function and death, or promotes cell survival remain to be elucidated. In this study we attempted to further define molecular events associated with the IH-associated apoptosis (Gozal et al., 2001a) and changes in protein expression (Gozal et al., 2002) in the CA1 region.

Cellular mRNAs are transcribed by the RNA polymerase II complex (RNAPII), in which the large subunit, Rpb1, has enzymatic activity. Rpb1 contains a long C-terminal domain (CTD) composed of 52 heptad repeats, each containing multiple serines that can undergo phosphorylation. Recently, we discovered that low-grade oxidative stress stimulates relocation of Rpb1 onto the DNA and hydroxylation of proline 1465 of Rpb1 (Mikhaylova et al., 2008) in the LGQLAP motif of Rpb1, a site involved in binding of the von Hippel-Lindau (pVHL)–associated E3 ubiquitin ligase (Kuznetsova et al., 2003). This hydroxylation is necessary for increased phosphorylation of serine 5 in C-terminal domain (Ser5) residues within the CTD (Mikhaylova et al., 2008) and is likely to be a crucial regulator of gene expression. Here we wanted to determine if IH, which is known to induce oxidative stress, regulates Rpb1 modifications in different regions of the brain.

We report that chronic IH, but not sustained hypoxia (SH), stimulated hydroxylation of proline 1465 in large subunit of RNA polymerase II (P1465) and phosphorylation of Ser5 of Rpb1, specifically in the CA1 region of the

*Correspondence to: M. F. Czyzyk-Krzeska, Department of Cancer and Cell Biology, The Vontz Center for Molecular Studies, University of Cincinnati College of Medicine, 3125 Eden Avenue, Cincinnati, OH 45267-0521, USA. Tel: +1-513-558-1957; fax: +1-513-558-5422.
E-mail address: Maria.Czyzykkrzeska@uc.edu (M. F. Czyzyk-Krzeska).

Abbreviations: CTD, C-terminal domain; Cul2, cullin 2; cyt, cytoplasmic extract; HA, hemagglutinin; HP, hydroxylated proline; IH, intermittent hypoxia; nuc, nuclear extract; PC12, pheochromocytoma; pVHL, von Hippel-Lindau protein; P1465, proline 1465 in large subunit of RNA polymerase II; RNAPII, RNA polymerase II; Rpb1, large subunit of RNA polymerase II; SDB, sleep disordered breathing; Ser5, serine 5 in C-terminal domain; SH, sustained hypoxia; VHL-AS, VHL antisense RNA; VHL-WT, wild type VHL.

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hippocampus and in the prefrontal cortex but not in other regions of the brain. Likewise, mice exposed to chronic IH demonstrated cognitive deficits related to dysfunction in those brain regions. Because phosphorylation of Rpb1 on Ser5 is crucial in the regulation of gene expression, induction of this modification of Rpb1 by IH could account for changes in the expression or function of numerous proteins in those important regions. Thus, we infer that observed biochemical changes in Rpb1 might underlie the behavioral changes that occur with IH.

EXPERIMENTAL PROCEDURES

Materials

The following commercially available antibodies were used: H14 (Covance, Berkeley, CA, USA); N20, C21 (Santa Cruz, Santa Cruz, CA, USA); pVHL (Ig32), anti-hemagglutinin (HA) tag (12CA5), and anti-cullin 2 (Cul2) (Fisher Sci., Fremont, CA, USA); and anti-Rbx1 (Invitrogen, Grand Island, NY, USA). The antibody against hydroxylated proline (HP) within the Rpb1 peptide was custom made by Alpha Diagnostic, Inc. (San Antonio, TX, USA). This antibody was used at a concentration of 1:500 for Western blots (Mikhaylova et al., 2008). Secondary antibodies were obtained from Sigma, St. Louis, MO, USA, Cell Signaling (Beverly, MA, USA), or Amersham (Piscataway, NJ, USA). Synthetic biotinylated peptides were made by Alpha Diagnostic, Inc.

Exposure of animals to IH and SH

Male C57Bl6 mice (Jackson Laboratories, Bar Harbor, ME, USA) were exposed to either chronic IH or SH for the indicated periods of time in commercially designed chambers (Oxycycler model A44XO, Biospherix, Redfield, NY, USA). The IH profile consisted of alternating room air and 7.8% oxygen every 150 s during daylight hours (7:00 AM to 7:00 PM) corresponding to the rest-and-sleep period of mice. The SH profile was programmed to continuously deliver 7.8% O_2 to the environment throughout the duration of the exposure. For the remaining 12 h of the sleep–wake cycle, the oxygen concentration was maintained at 21%. Control mice were exposed to circulating normoxic gas in a chamber identical to those housing IH- and SH-exposed mice. At the end of the exposure period (i.e. 14 days or 30 days), animals were sacrificed between 7:00 AM and 9:00 AM, i.e. 12–14 h after cessation of the corresponding hypoxic profile, and specific regions of the brain (hippocampus divided in CA1 and CA3 regions, prefrontal cortex, neocortex, and dorsocaudal brainstem) were surgically dissected. Mean wet tissue weights for individual tissue samples were 2–3 mg except for cortex samples, which weighed 5–6 mg. All animal experiments were performed according to protocols approved by IACUC of the University of Louisville and the University of Cincinnati. Every effort was made to minimize number of animals and their suffering.

Preparation of brain extracts

Nuclear extracts enriched for the chromatin fraction were obtained as follows: small pieces of specific frozen brain regions were first allowed to swell in cell lysis buffer for 10 min, and were then minced in the same buffer in a Dounce homogenizer to complete homogeneity. The homogenates were centrifuged at 14,000 rpm for 20 min, and the remaining pellets were extracted with 0.3 M NaCl for 30 min and digested with deoxyribonuclease and micrococcal nuclease (10 U and 37.5 U, respectively, per 100 μl of pellet volume) for 1 h to release DNA-bound RNAPII complexes. NP40 was added to a final concentration of 0.5%, and NaCl to a final concentration of 0.5 M, and the pellets were extracted for 30 min at 4 °C. For Western blotting, equal amounts of proteins were loaded in each lane and the samples were run on 4–20% gradient polyacrylamide gel electrophoresis using standard protocols.

Place navigation/spatial reference task in the Morris water maze

The Morris water maze was configured to test performance that reflects types of learning and memory traditionally defined as spatial reference, as previously described (Gozal et al., 2001; Kheirandish et al., 2005). In brief, 1 day prior to place learning, mice exposed to either IH, SH, or control normoxic conditions for 14 or 30 days were habituated to the water maze during a free swim. Place learning was assessed over 6 days of consecutive training sessions during which mice continued to be exposed to their respective hypoxia treatments. We used a spaced training regimen that has been demonstrated to elicit optimal learning in mice (Gerlai and Clayton, 1999). Each place-training session consisted of three trials separated by a 10-min intertrial interval, and such sessions occurred between 7:30 PM and 10:30 PM. At daily sessions, each animal was placed into the pool from four quasirandom start points and allowed a maximum of 90 s to escape to the platform where it was allowed to remain for 15 s. Mice that failed to escape were led to the platform. The position of the platform remained constant across trials. Twenty-four hours following the second, fourth, and final (6th) training session, the platform was retracted for a 30-s probe trial during which the time spent in each of the four quadrants of the maze and the number of target crossings and proximity to the previous location of the platform were recorded. Probe trials provide a measure of spatial bias developed during learning. To assess performance during place training, mean escape latencies, swim distances, and swim speeds were analyzed by two-way analysis of variance with repeated measures on block (three trials), followed by Newman-Keuls tests when appropriate. To assess probe performance, mean quadrant times, target crossings, and time spent in the general vicinity of the platform location were compared as well.

Cell culture and exposure of cells to IH or SH

Pheochromocytoma (PC12) cells overexpressing human HA-tagged pVHL, or expressing rat VHL antisense RNA (VHL-AS) were grown as described earlier (Kuznetsova et al., 2003). Experiments were timed so that the cells were 70% to 90% confluent at the time of collection. Cells were exposed to the indicated durations of repeated fluctuations of O_2 from 30% to 1% O_2 (in constant 5% CO_2) in a hypoxic workstation (Coy Laboratory Products, Grass Lake, MI, USA). The IH cycle was created using the manufacturer-supplied computer program to allow infusion of N_2 into the chamber until O_2 reached 1% in the tissue culture media in a control plate carrying the same number of cells, followed by an infusion of O_2 until the oxygen level measured in the culture media was 30%. The O_2 concentrations were determined in the chamber air using an O_2 sensor and monitor provided with the hypoxia workstation, and O_2 in the media was measured using a Dissolved Oxygen Meter (World Precision Instruments, Sarasota, FL, USA). Both measurements were performed for the entire duration of each exposure. The exposure to SH was achieved by putting cells into the hypoxic workstation with O_2 equilibrated to 1%. Full equilibration in the culture medium was achieved after 30 min of exposure. Extracts from PC12 cells were obtained as described before (Kuznetsova et al., 2003).

In vitro pVHL-peptide binding reaction

Ten micrograms of biotinylated peptide were incubated with streptavidin-coated Dynabeads (M-280, Dynal, Invitrogen, Carlsbad, CA, USA) in 300 μl of buffer containing 20 mM Tris, pH 8, 100 mM NaCl, 0.5% NP-40, and 1 mM EDTA for 1 h at room temperature. Washed