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Chronic hyperoxia alters the expression of neurotrophic factors in the carotid body of neonatal rats

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Abstract

Chronic exposure to hyperoxia alters the postnatal development and innervation of the rat carotid body. We hypothesized that this plasticity is related to changes in the expression of neurotrophic factors or related proteins. Rats were reared in 60% O₂ from 24–36 h prior to birth until studied at 3 d of age (P3). Protein levels for brain-derived neurotrophic factor (BDNF) were significantly reduced (–70%) in the P3 carotid body, while protein levels for its receptor, tyrosine kinase B, and for glial cell line derived neurotrophic factor (GDNF) were unchanged. Transcript levels in the carotid body were downregulated for the GDNF receptor Ret (–34%) and the neuropeptide Vgf (–67%), upregulated for Cbln1 (+205%), and unchanged for Fgf2; protein levels were not quantified for these genes. Immunohistochemical analysis revealed that Vgf and Cbln1 proteins are expressed within the carotid body glomus cells. These data suggest that BDNF, and perhaps other neurotrophic factors, contribute to abnormal carotid body function following perinatal hyperoxia.

Keywords

control of breathing; peripheral chemoreceptor; neuroplasticity; neurotrophin; mRNA expression; protein expression

1. Introduction

Chronic exposure to moderate hyperoxia during the early postnatal period impairs development of the respiratory control system. Hyperoxia-treated rats tend to have lower resting ventilation and more variable breathing patterns as neonates (Bavis et al., 2010), and adult rats exhibit a diminished hypoxic ventilatory response (HVR) following exposure to 30–60% O₂ for the first 1–4 postnatal weeks (Ling et al., 1996; Bavis et al., 2003). The long-lasting attenuation of the HVR after chronic postnatal hyperoxia is primarily explained by abnormal development of the carotid body and its chemoafferent pathway (Ling et al., 1997; Bavis, 2005). The carotid bodies of hyperoxia-treated rats are significantly smaller than those of age-matched controls due to decreased cell division (Erickson et al., 1998; Wang & Bisgard, 2005; Broge et al., 2009). In addition, Erickson et al. (1998) observed fewer unmyelinated axons in the carotid sinus nerve (CSN) and fewer dopaminergic neurons in the petrosal ganglion of hyperoxia-treated rats, suggesting degeneration of carotid chemoafferent neurons. Importantly, removal of the carotid body during the early postnatal

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period prompts degeneration of primary sensory neurons within the petrosal ganglion, suggesting that carotid body cells provide trophic support to these neurons (Hertzberg et al., 1994). Collectively, these observations suggest that hyperoxia might influence the expression of molecules that regulate postnatal growth and innervation of the carotid body.

Neurotrophins such as brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) are important regulators of the morphological and functional development of the nervous system (Huang & Reichardt, 2001). Importantly, both BDNF and GDNF are critical to the prenatal and postnatal development of the carotid body and its innervation *in vivo* (Katz, 2003, 2005). BDNF and GDNF null mutant mice exhibit depressed and irregular breathing during normoxia as well as frequent apneas (Erickson et al., 1996, 2001), while BDNF mutants also display reduced peripheral O₂ chemosensitivity (Erickson et al., 1996). Moreover, newborn mice deficient in BDNF, GDNF, and/or TrkB (the high-affinity receptor for BDNF) also exhibit severe reductions in the number of dopaminergic neurons in the nodose-petrosal ganglion (Erickson et al., 1996, 2001), consistent with the loss of carotid chemoafferent neurons. The presence of TrkB in carotid body glomus cells suggests that these neurotrophins may also serve autocrine and/or paracrine roles in the survival, growth and maturation of these cells (Wang & Bisgard, 2005; Izal-Azcárate et al., 2008). Thus, to explain the effects of hyperoxia on carotid body development, we hypothesized that BDNF, GDNF, and/or their receptors (TrkB and Ret, respectively) would be downregulated by chronic hyperoxia. Because deleterious effects on carotid body morphology and physiology are well developed within 4–5 days of postnatal hyperoxia (Broge et al., 2009; Donnelly et al., 2009), we predicted that changes in gene expression causally linked to this plasticity would already be evident at 3 d of age.

Many neurotrophic factors in addition to BDNF and GDNF are expressed in the carotid body (Izal-Azcárate et al., 2008; Porzionato et al., 2008). Therefore, a secondary objective was to determine whether hyperoxia also influences mRNA expression of additional neurotrophic factors or related molecules. The protein encoded by Fgf2, also known as basic FGF, is expressed in carotid body glomus cells (Paciga & Nurse, 2001; Izal-Azcárate et al., 2008) and influences the survival and proliferation of glomus cell cultures *in vitro* (Porzionato et al., 2008). Based on these data, we hypothesized that postnatal hyperoxia would downregulate Fgf2 in the carotid body. Results from a preliminary PCR array analysis (E.F. Dmitrieff & R.W. Bavis, unpublished observations) prompted us to further hypothesize that postnatal hyperoxia would downregulate VGF nerve growth factor inducible (Vgf) and upregulate cerebellin 1 precursor (Cbln1). The expression of Cbln1 or Vgf has not previously been reported in the carotid body. Cbln1 is a secreted glycoprotein linked to synaptic connectivity and plasticity in the central nervous system (CNS), particularly through its interaction with the $\delta 2$ glutamate receptor (GluR $\delta 2$) in the cerebellum (Yuzaki, 2009, 2010). Vgf is widely expressed in the CNS and endocrine tissues, and the neuropeptides derived from Vgf have been linked to the regulation of energy balance and reproduction (Jethwa & Ebling, 2008) as well as neurogenesis and BDNF-dependent synaptic plasticity within the CNS (Thakker-Varia & Alder, 2009).

2. Methods

2.1 Experimental animals

Timed pregnant SASCO Sprague-Dawley rats were obtained from Charles River Laboratories (Colony P04, Portage, MI, USA). All rats were maintained on a 12-h light:12-h dark cycle with food and water *ad libitum* throughout the study. On gestational day 20 (24–36 h prior to birth), dams were placed into an acrylic chamber flushed with a mixture of O₂ and air so as to maintain chamber gas concentrations at 60% O₂ and <0.4% CO₂; the resulting litters (“Hyperoxia”) were maintained under these conditions until studied. To

serve as controls, additional litters (“Normoxia”) were placed into an acrylic chamber flushed with air (21% O₂, <0.4% CO₂) from gestational day 20. Due to space limitations in the TrkB protein study, however, control rats for this experiment were raised in the same room as Hyperoxia rats, but outside the chamber; there are no detectable differences in HVR between control rats maintained in a chamber flushed with room air and those maintained in open room air (Bavis et al., 2007). Litters were maintained at their respective gas concentrations until carotid body collection at 3 days of age (P3, day of birth = P0). Pups were kept with their mothers until tissue collection to minimize any effects of maternal separation. Chambers were opened briefly (<5 min) to remove litters for carotid body collection and to clean cages as needed.

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Bates College.

2.2 mRNA Expression Analysis

Total RNA was isolated from neonatal rat carotid bodies to assess the effects of hyperoxia on mRNA expression for several genes of interest (BDNF, GDNF, Ret, TrkB, Cbln1, Fgf2, and Vgf). To accomplish this, tissue was collected in three separate rounds of experiments (experiment 1: BDNF, GDNF, Ret; experiment 2: TrkB, Cbln1, Fgf2; experiment 3: Vgf). Efforts to increase the rate of tissue collection and optimize cDNA yields in successive experiments resulted in minor procedural differences among the three rounds (see below). These differences should not impact the conclusions of this study, however, since comparisons between Normoxia and Hyperoxia groups for each gene of interest are restricted to samples collected in the same experiment (and thus handled the same way).

2.2.1 Carotid Body Collection—For BDNF, GDNF, and Ret, P3 pups were killed via intercardiac injection of Beuthanasia-D (Schering-Plough Animal Health Corp., Union, NJ, USA) (0.05 ml/10 g). The right and left carotid bifurcations were rapidly harvested and placed in RNeasy lysis buffer (Qiagen, Austin, TX, USA). Carotid bodies were then dissected out and placed in 350 µl RNeasy lysis buffer and frozen at −20°C. In subsequent experiments, P3 pups were decapitated, and carotid bodies were harvested and either placed in 350 µl RNeasy lysis buffer and frozen at −20°C (TrkB, Cbln1, Fgf2) or flash frozen on dry ice and stored at −80°C (Vgf). Due to the small size of the neonatal carotid body, each sample consisted of carotid bodies pooled from one litter (14 – 26 carotid bodies per pool). In each of the three experiments, carotid bodies were collected from a total of 6 or 7 litters per treatment group (i.e., n = 6–7 independent samples per treatment).

2.2.2 RNA Isolation and cDNA Synthesis—In each of the three experiments, pooled carotid bodies were homogenized for 20 s in RLT lysis buffer (Qiagen, Valencia, CA, USA), and RNA was extracted from the homogenates using an RNeasy Micro RNA Isolation Kit (Qiagen). RNA quantity and quality were assessed using an RNA 6000 Nano Chip in a Bioanalyzer (Agilent, Santa Clara, CA, USA). Total RNA yield averaged 0.6 µg per sample (30 ng per carotid body).

For BDNF, GDNF, and Ret, first strand cDNA was synthesized from 150 ng total RNA using 200U Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), 1 µl primer mix (200 ng/µl oligo dT's and 50 ng/µl random hexamers) (Promega, Madison, WI, USA), 1 µl 10 mM dNTPs (Promega), 4 µl 5× First strand buffer (Invitrogen), 2 µl 0.1 M dithiothreitol (DTT) (Invitrogen), and 1 µl RNaseOUT (Invitrogen); these reactions were incubated at 25°C for 5 min followed by 42°C for 50 min and 70°C for 15 min. For TrkB, Cbln1, and Fgf2, cDNA was synthesized from 200 ng total RNA using 1 µl ImProm-II Reverse Transcriptase (Promega), 1 µl primer mix (200 ng/µl oligo dT's and 50 ng/µl

random hexamers) (Promega), 1 μ l 10 mM dNTPs (Promega), 4 μ l ImProm-II 5 $\times$ First strand buffer (Promega), 2 μ l 25 mM MgCl₂ (Promega), and 1 μ l RNaseOUT (Invitrogen); these reactions were incubated at 25°C for 5 min followed by 42°C for 60 min and 70°C for 15 min. For Vgf, cDNA was synthesized from 300 ng total RNA using RT² First Strand Kit (SABiosciences, Frederick, MD USA) according to kit instructions with the exception of replacing GE Buffer with water. The resulting single-stranded cDNA products were stored at -20°C until quantitative PCR analysis.

2.2.3 Quantitative RT-PCR—Each gene of interest was assayed on a separate 96-well plate, with individual Normoxia and Hyperoxia cDNA samples run in triplicate for the corresponding gene of interest and for β -actin. Preliminary analysis indicated that β -actin was the most stable reference gene compared to tyrosine hydroxylase, synaptophysin, chromogranin A, and β -III tubulin (geNORM v. 3.5, Ghent University Hospital Center for Medical Genetics, Belgium; data not shown). Primers were designed using NetPrimer (PRIMER Biosoft International, Palo Alto, CA, USA), with the exception of β -actin (103) and BDNF, for which sequences were kindly provided by Dr. Gordon Mitchell at the University of Wisconsin-Madison School of Veterinary Medicine. All primer pairs (Table 1) were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

Amplification was performed using Brilliant SYBR Green QPCR Master Mix (Stratagene, Cedar Creek, TX, USA) in an Mx3000 qPCR System (Stratagene) as follows: 95°C $\times$ 10 min, 40 cycles of 95°C $\times$ 30 s, 58/60/65°C $\times$ 1 min, 72°C $\times$ 30 s (see Table 1 for annealing temperatures for individual assays). Dissociation curve analyses were run to confirm a single gene product in each well, and “no template” and “no reverse transcriptase” controls were included to monitor for genomic DNA and other contamination.

For each cDNA sample, triplicate C_T values were averaged to produce one value for the gene of interest and for β -actin. C_T values for the gene of interest were then normalized to β -actin C_T values using the 2^{- $\Delta\Delta$ CT} method (Livak and Schmittgen, 2001). The averaged C_T values from the Normoxia samples were used as the calibrator for that plate, and the data are reported relative to that value (i.e., percent of Normoxia).

2.3 Protein Expression Analysis

2.3.1 Carotid Body Collection—For enzyme-linked immunosorbent assays (ELISA) and western blot analyses, P3 rats were decapitated, and both carotid bifurcations were rapidly harvested and placed into ice cold Ringer's Solution (in mM: 125 NaCl, 5 KCl, 4.8 CaCl₂, 1.2 Na₂HPO₄, 26 NaHCO₃, 1.2 MgSO₄, and 60 dextrose, pH = 7.35–7.40). Carotid bodies were then dissected out, flash frozen on dry ice, and stored at -80°C. Each sample consisted of carotid bodies pooled from one litter (ELISA; 14 – 26 carotid bodies per pool) or two litters (western blot; 28 – 52 carotid bodies per pool). Carotid bodies were collected from a total of 6 litters per treatment for the BDNF ELISA, 8 litters per treatment for the GDNF ELISA, and 12 litters per treatment for the TrkB western blot (i.e., n= 6, 8, and 6 independent samples per treatment group, respectively).

2.3.2 ELISA—BDNF and GDNF protein (free, mature isoforms) were measured by ELISA. The BDNF and GDNF E_{max} immunoassays (Promega) were performed according to the manufacturer's protocol with the exception of an increase in the primary antibody concentrations to amplify the signal: anti-BDNF monoclonal antibody was doubled in the BDNF assay for a final dilution of 1:500; anti-GDNF monoclonal antibody and anti-Human GDNF polyclonal antibody were both doubled in the GDNF assay for final dilutions of 1:500 and 1:250, respectively. The acid treatment procedure was omitted. Carotid bodies were homogenized for 20 s in 250 – 300 μ l of lysis buffer (137 mM NaCl, 20 mM Tris-HCl

(pH 8.0), 1% v/v Igepal, 10% v/v glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM sodium vanadate, 10 µg/ml aprotinin, 1 µg/ml leupeptin). The homogenates were centrifuged for 10 min at $3000 \times g$ at 4°C and stored on ice until assayed in duplicate on the ELISA plate (<30 min). Total protein was assayed in duplicate on 12-fold diluted homogenates using a Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer's microplate protocol. Absorbances for the ELISA and total protein reactions were measured using a Synergy HT microplate reader with KC4 software (Bio-Tek, Winooski, VT, USA). Duplicate values were averaged to produce a single value for each sample, and the BDNF and GDNF concentrations were normalized to total protein. Total protein yield averaged 15.3 µg per sample (0.8 µg per carotid body).

2.3.3 Western Blot—TrkB protein was measured by western blot. Carotid bodies were homogenized in 150 µl of RIPA lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Igepal, 1% sodium deoxycholate, 0.1% SDS) (Pierce Technology) containing 2% protease inhibitors (Sigma-Aldrich) and left to sit on ice for 30 min. The homogenates were centrifuged at $10,000 \times g$ for 60 min at 4°C, and the pellets were discarded. Total protein was assayed on 10-fold diluted homogenates using a Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer's microplate protocol, and all homogenates were then diluted to the same total protein concentration. Total protein yield averaged 13.3 µg per sample (0.4 µg per carotid body). Tissue homogenates (60 ng total protein) were boiled for 3 min in 2× SDS-PAGE Laemmli buffer (Bio-Rad, Hercules, CA, USA) containing 5% β-mercaptoethanol and electrophoretically separated on a gradient (4–20%) Tris HCl gel (Bio-Rad) in 0.1% SDS running buffer at 200V. Gels were equilibrated in blotting buffer (25 mM Tris base, 40 mM glycine, 10% methanol, pH 9.4) for 10 min and subjected to electroblotting onto Immobilon-P polyvinylidene fluoride (PVDF) membrane (1.2 mAmp/cm^2). The membrane was then blocked for 1 h in 10% non-fat milk in TBST (25 mM Tris base, 150 mM NaCl, 0.1% Tween-20) and incubated overnight at 4°C with a TrkB primary antibody that recognizes the truncated form of the TrkB protein (ab51190, 1:1000; Abcam, Cambridge, MA, USA). Following washing, membranes were treated for 1 h with a secondary antibody conjugated to alkaline phosphatase (sc-2021, 1:10000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, the blot was developed with ECF substrate (Amersham Biosciences, Piscataway, NJ, USA), and fluorescent images were scanned using a Fuji FLA-5000 phosphorimager. The membrane was then stripped in 2% SDS, 100 mM β-mercaptoethanol, 50 mM Tris, pH 6.8 at 50°C for 30 min, washed, and reprobed with β-actin primary antibody (ab8227, 1:2500; Abcam) following the same protocol as for TrkB. Western blots were analyzed using Adobe Photoshop (San Jose, CA, USA). Three separate density measurements were calculated for each band. Relative density values for each sample were calculated as a ratio of averaged TrkB band density to averaged β-actin density minus the measured background density. The investigator was blind to the treatment group during all quantification steps.

2.4 Immunohistochemistry

To localize expression of Cbln1 and Vgf in the carotid body, P3 pups were decapitated, and carotid bifurcations were harvested and post-fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 1 h and cryoprotected in sucrose (1 d in 30% sucrose in 0.1 M PBS, pH 7.4). Carotid bifurcations were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA, USA) on dry ice and stored at −80°C until sectioning (9 µm) with a cryostatic microtome onto Superfrost Plus slides (Fisherbrand, Pittsburgh, PA, USA). Slides were stored at −80°C until immunohistochemical analysis.

Sections were incubated in 0.2% Triton X-100 in PBS to permeabilize cell membranes and blocked in 10% BlokHen (Aves Labs, Tigard, OR, USA)/10% Normal Donkey Serum

(Jackson ImmunoResearch, West Grove, PA, USA). Sections were labeled with either tyrosine hydroxylase (TH) as a marker for glomus (type I) cells or glial fibrillary acidic protein (GFAP) as a marker for sustentacular (type II) cells and then co-labeled with either Cbln1 or Vgf. Sections were incubated overnight at 4°C with the following primary antibodies: Cbln1 (LS-B478, 1:100; LifeSpan Biosciences, Seattle, WA, USA); TH (TH, 1:1000; Aves Labs); GFAP (GFAP-S, 1:100; Aves Labs); Vgf (ab69989, 1:100; Abcam). Sections were incubated for 1 h with secondary antibodies conjugated to TRITC for Cbln1 and Vgf (sc-2095, 1:100; Santa Cruz Biotechnology) or FITC for TH and GFAP (F-1005, 1:500; Aves Labs). Slides were coverslipped with Vectashield HardSet mounting medium + DAPI (Vector Labs, Burlingame, CA, USA) and imaged with Nikon's NIS Elements software using a Nikon Eclipse 80i microscope with a 2 megapixel camera.

2.5 Statistical Analysis

In most cases, protein (concentration or density values) and mRNA ($2^{-\Delta\Delta CT}$ values) expression levels were compared between treatment groups (Hyperoxia vs. Normoxia) using independent sample *t*-tests. However, treatment groups were compared using the Mann-Whitney U test when variances differed between groups (i.e., TrkB and Cbln1 mRNA assays). All statistical tests were run using GraphPad Prism v 5.00 (GraphPad Software, La Jolla, CA USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. BDNF, GDNF, and their receptors

BDNF mRNA expression in the carotid body of P3 rats was not affected by chronic hyperoxia (Fig. 1a). In contrast, the BDNF protein concentration was reduced by 70% relative to carotid bodies collected from age-matched Normoxia rats ($P = 0.025$; Fig. 1b). The mRNA expression for TrkB, the receptor for BDNF, was increased by approximately 120% ($P = 0.018$; Fig. 2a), but no corresponding change in TrkB protein expression was detected by western blot analysis (Fig. 2b,c). Hyperoxia did not change the expression levels for GDNF mRNA (Fig. 3a) or protein (Fig. 3b). A modest, 34% decrease in Ret mRNA expression was detected ($P = 0.008$; Fig. 4).

3.2. Fgf2, Cbln1, and Vgf

The expression of Fgf2, Cbln1, and Vgf mRNA was assessed by quantitative RT-PCR (Fig. 4). There was no evidence for a change in Fgf2 mRNA expression. Transcripts for both Cbln1 and Vgf were detected in the carotid bodies of P3 rats. Cbln1 expression was strongly upregulated by chronic hyperoxia, with mRNA expression increased by 205% compared to age-matched Normoxia rats ($P = 0.002$). In contrast, Vgf mRNA expression was decreased by 67% ($P = 0.003$). No attempt was made to quantify protein expression for Cbln1 or Vgf.

To our knowledge, Cbln1 and Vgf have never been reported to be expressed in the carotid body. Therefore, we used immunohistochemistry to determine which cell types express these proteins in the carotid body of P3 Normoxia rats (Fig. 5). Both Cbln1 and Vgf co-localized with TH, indicating that they are expressed in glomus (type I) cells. In contrast, neither Cbln1 nor Vgf co-localized with GFAP, suggesting that they are not expressed in the sustentacular (type II) cells.

4. Discussion

Previous studies revealed that chronic exposure to moderate hyperoxia alters the morphological development and innervation of the carotid body in rats, primarily within the first postnatal week (Erickson et al., 1998; Bisgard et al., 2003; Wang & Bisgard, 2005). Our

results are consistent with the hypothesis that altered expression of neurotrophic factors contributes to this plasticity. Exposure to 60% O₂ significantly reduced the levels of BDNF protein, but not of GDNF, in the carotid body by P3. Expression of mRNA for TrkB was increased, but protein levels were unchanged. Changes in mRNA expression for Ret, Cbln1, and Vgf were also detected, suggesting additional pathways by which hyperoxia might influence carotid body development. Although Fgf2 influences glomus cell survival and proliferation in culture (Porzionato et al., 2008), we did not detect any change in mRNA expression for this gene.

4.1 Critique of methods

We studied mRNA and protein isolated from the whole carotid body. The carotid body is comprised of several cell types, including the O₂-sensitive glomus (type I) cells, sustentacular (type II) cells, afferent and efferent neurons, and vascular endothelial cells, and the genes investigated in the present study are expressed differentially among these cell types. For example, BDNF, GDNF, Fgf2, Cbln1, and Vgf are expressed in glomus cells but not sustentacular cells in the neonatal rat carotid body (Wang & Bisgard, 2005; Izal-Azcárate et al., 2008; Porzionato et al., 2008; present study). Given that hyperoxia alters carotid body size, it is possible for disproportionate changes in cell numbers among the different cell types to alter whole-carotid body protein or transcript levels without changing actual gene expression within individual cells. This seems unlikely to explain the changes in protein and mRNA expression observed in the present study, however. First of all, decreases in carotid body size reflect proportionate decreases in the volume occupied by glomus cells and non-glomic tissue (Erickson et al., 1998). Moreover, if the observed mRNA and protein expression changes were an artifact, we would have expected decreased expression for all glomus cell-specific molecules (versus some decreasing, some increasing, and some remaining unchanged as observed here).

We may have overlooked additional changes in gene expression by limiting hyperoxic exposures to 3 d in the present study. However, the early onset of changes in BDNF protein levels and in transcript levels for other genes (Ret, Cbln1, Vgf) correlates well with the fact that morphological (Broge et al., 2009) and physiological (Donnelly et al., 2009; Bavis et al., 2010) carotid body plasticity are already well established within 4–5 d of postnatal hyperoxia.

4.2. BDNF and GDNF signaling in hyperoxia-induced carotid body plasticity

Carotid body BDNF protein was markedly reduced (–70%) in rat pups following only a 3-d exposure to 60% O₂. This result was recently confirmed by Mason et al. (2010) who, in a conference abstract, reported a 90% decrease in carotid body BDNF protein in rats following a 7-d exposure to 60% O₂. Although we did not observe a change in BDNF mRNA levels, it is possible that hyperoxia influences the translation of BDNF mRNA or the rate of protein degradation. Interestingly, this is not the first study to observe a disparity between changes in BDNF mRNA and protein expression (e.g., Peiris et al., 2004; Bairam et al., 2010).

While the present data do not provide direct evidence that reduced carotid body BDNF protein causes the respiratory changes observed after chronic postnatal hyperoxia, several lines of evidence support this conclusion. First of all, hyperoxia-treated neonatal rats exhibit respiratory phenotypes similar to those of BDNF null mutant mice (Erickson et al., 1996), including more variable breathing patterns, reduced normoxic ventilation, decreased peripheral chemosensitivity, and loss of TH-positive neurons in the petrosal ganglion (Erickson et al., 1998; Donnelly et al., 2005, 2009; Bavis et al., 2010). Secondly, there are similar critical periods for hyperoxia-induced plasticity (first two postnatal weeks; Erickson

et al., 1998; Bavis et al., 2002) and the dependence of chemoafferent neurons on carotid body-derived trophic support *in vivo* (<3 postnatal weeks; Hertzberg et al., 1994). Finally, it has been proposed that at least some of the effects of hyperoxia on respiratory control development are linked to carotid body inactivity (Ling et al., 1997; Bavis, 2005; Bavis et al., 2007). Indeed, high arterial PO₂ levels are expected to decrease spontaneous carotid body depolarization, thereby altering activity-dependent gene expression and/or intercellular signaling. Likewise, the transcription, translation, and release of BDNF are regulated in an activity-dependent manner in many neurons (Brady et al., 1999; Balkowiec & Katz, 2000; Loebrich & Nedivi, 2009; Cohen-Cory et al., 2010; Lau et al., 2010), and the same could be true for other cell types (e.g., glomus cells). BDNF also modulates activity-dependent postnatal maturation of carotid chemoafferent neurons (Katz, 2005).

Importantly, it does not appear that TrkB receptor expression compensates for decreases in BDNF protein in the carotid body. Although we did detect an increase in the mRNA for TrkB, this was not associated with any change in TrkB protein expression. Similarly, Mason et al. (2010) reported no change in carotid body TrkB expression after 7-d hyperoxic exposures.

While GDNF appeared to be a good candidate to mediate hyperoxia-induced carotid body plasticity, we did not detect any change in GDNF mRNA or protein after 3 d of hyperoxia. There was a modest reduction in mRNA for the GDNF receptor Ret (−34%), however, suggesting that the GDNF signaling pathway is affected by hyperoxia. Unfortunately, and despite several attempts, we were unable to reliably detect Ret protein expression in the P3 carotid body by western blot (J.T. Wilson and R.W. Bavis, unpublished observations), perhaps because Ret expression is restricted to nerve endings (a very small proportion of the carotid body volume) in the neonatal carotid body (Wang & Bisgard, 2005).

4.3 Cbln1 and Vgf mRNA expression in the carotid body

Our data indicate that Cbln1 and Vgf mRNA are expressed in the neonatal carotid body, and immunohistochemistry revealed that the corresponding proteins are expressed in the glomus cells, but not sustentacular cells. Hyperoxia increased transcript levels for Cbln1 and decreased transcript levels for Vgf in the neonatal carotid body. Although it remains to be determined whether hyperoxia causes corresponding changes in protein expression, these observations suggest that these proteins could play a role in hyperoxia-induced plasticity. Neither of these proteins has previously been reported to be expressed in the carotid body or in any region associated with respiratory control, so it is difficult to speculate on their role in hyperoxia-induced carotid body plasticity. Both Cbln1 and Vgf appear to contribute to the induction, maintenance, and functional plasticity of synapses in the CNS (Alder et al., 2003; Bozdagi et al., 2008; Thakker-Varia & Alder, 2009; Yuzaki, 2009, 2010), and therefore might influence synaptic connectivity in the carotid body. Cbln1 may also regulate cell proliferation, with increased expression of Cbln1 being linked to hypoplasia in the adrenal gland (Hóchol et al., 2001; Takahashi et al., 2002).

Like BDNF, it is possible that hyperoxia regulates Cbln1 and Vgf expression through activity-dependent mechanisms. Chronic activation of cerebellar granule cells leads to decreased Cbln1 mRNA and protein within hours (Iijima et al., 2009). If the opposite is also true (i.e., that reduced neuronal activity increases Cbln1 expression), hyperoxia-induced carotid body inactivity could explain the elevated Cbln1 mRNA levels detected in the present study. Vgf expression is also influenced by neuronal activity (Snyder et al., 1998). In addition, Vgf and BDNF appear to be interrelated in the CNS, with Vgf often being co-expressed with TrkB (Snyder et al., 1997). BDNF induces Vgf transcription (e.g., Alder et al., 2003; Cazzin et al., in press), and Vgf may in turn modulate BDNF release in the

hippocampus (Bozdagi et al., 2008). Thus, hyperoxia could reduce Vgf mRNA expression in the neonatal carotid body through its effects on BDNF levels.

4.4 Summary

In conclusion, these observations collectively support a model in which chronic postnatal exposure to moderate hyperoxia alters the development of respiratory control in part via changes in the expression of neurotrophic factors in the carotid body. These changes are likely mediated through activity-dependent regulation of BDNF (and possibly Cbln1 and Vgf). Specifically, reduced BDNF expression and/or release by glomus cells during hyperoxia would lead to the degeneration and/or abnormal maturation of chemoafferent neurons (Hertzberg et al., 1994; Brady et al., 1999). While the direct effects of BDNF on glomus cell growth and proliferation have not been studied, it is possible that decreased signaling (autocrine or paracrine) through TrkB receptors on the glomus cell membrane may contribute to carotid body hypoplasia. If decreased expression in Ret mRNA leads to reduced numbers of functional GDNF receptors, inadequate GDNF signaling might be involved as well. However, since the same carotid chemoafferent neurons that depend on GDNF also depend on BDNF (Erickson et al., 2001), the decrease in BDNF availability may be sufficient to explain the resulting phenotype.

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Fig. 1. BDNF expression in the carotid bodies of normoxic and hyperoxic P3 rats. (A) BDNF mRNA levels (normalized to β -actin) measured by quantitative RT-PCR (n=6 per treatment group). (B) BDNF protein concentration (normalized to total protein) measured by ELISA (n=6 per treatment group). Values are mean $\pm$ SEM. * P <0.05 vs. Normoxia.

Fig. 2.

TrkB expression in the carotid bodies of normoxic and hyperoxic P3 rats. (A) TrkB mRNA levels (normalized to β -actin) measured by quantitative RT-PCR (n=7 per treatment group). (B) Representative samples from the western blots of TrkB (92 kD) and β -actin (42 kD, loading control). Note: Gamma levels were decreased in order to better visualize bands. (C) Relative density of TrkB western blot bands (normalized to β -actin) (n=6 per treatment group). Values are mean $\pm$ SEM. * P <0.05 vs. Normoxia.

Fig. 3. GDNF expression in the carotid body of normoxic and hyperoxic P3 rats. (A) GDNF mRNA levels (normalized to β -actin) measured by quantitative RT-PCR (n=6 per treatment group). (B) GDNF protein levels (normalized to total protein) measured by ELISA (n=8 per treatment group). Values are mean $\pm$ SEM. No significant differences were detected between treatment groups.

Fig. 4. Quantitative RT-PCR analysis of Fgf2, Cbln1, Vgf, and Ret mRNA levels (normalized to β -actin) in the carotid bodies of normoxic and hyperoxic P3 rats (n=6 per treatment group). Values are mean $\pm$ SEM. ** $P < 0.01$ vs. Normoxia.

Fig. 5. P3 rat carotid body sections processed for double immunofluorescence. (A) Cbln1 (red, left), GFAP (green, center), and merged (right). (B) Cbln1 (red, left) and TH (green, center), and merged (right). (C) Vgf (red, left), GFAP (green, center), and merged (right). (D) Vgf (red, left), TH (green, center), and merged (right). Scale bar = 100 μ m.

Table 1

Primers used for quantitative RT-PCR.

Target Gene	Forward Primer (5'-3')	Conc. (nM)	Reverse Primer (5'-3')	Conc. (nM)	Expected Length (bp)	Annealing Temp (°C)
β -actin ^a	CTAAGGCCAACCGTGAAAAGAT	600	CCAGAGGCATACAGGGACAAC	100	103	60/65
β -actin ^b	GGGTGTGATGGTGGGTAT	300	CATTGTAGAAAAGTGTGGTGC	300	155	58
BDNF	CTGACACTTTTGTGACACGTGATC	600	AGGCTCCAAAAGGCACCTTGACT	100	134	65
GDNF	GGTCACACAGATAAACAAAGCG	600	TAGCCCAAAACCCAAAGTCA	600	173	65
TrkB	CCTTGACTTGTCTGACCTGA	300	GGGTATTCTTGTGCTGCTCTC	300	143	60
Ret	GCACAGCTCTGCTCTATGTCC	300	GAGCTGCTCCAGGAACCTATG	300	188	65
Cbln1	ACAGACCATCCAGGTGAGC	300	GGAATCCAGAGAAAGGTTGAGTA	300	194	65
Vgf	GACTTTGACACCCCTTATCCA	300	CTCCCTGCCCTCTACCTGA	300	146	60
Fgf2	GGCTATGAAGGAAGATGGA	300	ATTGGAAGAAACAGTATGGC	300	204	58

^a used as reference gene for all assays except Fgf2

^b used as reference gene for Fgf2 assay to accommodate a lower annealing temperature