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Time-course of alterations in pre- and post-synaptic chemoreceptor function during developmental hyperoxia

David F. Donnelly^{a,*}, Ryan W. Bavis^b, Insook Kim^c, Hassan A Dbouk^c, and John L. Carroll^c

^aDepartment of Pediatrics, Division of Respiratory Medicine, Yale University School of Medicine, New Haven, CT 06520

^bDepartment of Biology, Bates College, Lewiston, ME 04240 USA

^cDepartment of Pediatrics, Division of Pulmonary Medicine, University of Arkansas for Medical Sciences, Little Rock, Arkansas, 72202 USA

Abstract

Postnatal hyperoxia exposure reduces the carotid body response to acute hypoxia and produces a long-lasting impairment of the ventilatory response to hypoxia. The present work investigated the time-course of pre- and post-synaptic alterations following exposure to hyperoxia ($F_{iO_2}=0.6$) for 1, 3, 5, 8 and 14 days (d) starting at postnatal day 7 (P7) as compared to age-matched controls. Hyperoxia exposure for 1d enhanced the nerve response and glomus cell calcium response to acute hypoxia, but exposure for 3-5d caused a significant reduction in both. Hypoxia-induced catecholamine release and nerve conduction velocity were significantly decreased by 5d hyperoxia. We conclude that hyperoxia exerts pre-synaptic (glomus cell calcium and secretory responses) and post-synaptic (afferent nerve excitability) actions to initially enhance and then reduce the chemoreceptor response to acute hypoxia. The parallel changes in glomus cell calcium response and nerve response suggest causality between the two and that environmental hyperoxia can affect the coupling between acute hypoxia and glomus cell calcium regulation.

Keywords

carotid body; chemoreceptors; hypoxia; calcium; hyperoxia

1. Introduction

Carotid body chemoreceptors are the principal sensors for detecting systemic hypoxia and initiating an increased drive to breathe, arousal from sleep and sympathetic stimulation. They undergo major developmental changes at the time of birth and in the immediate post-natal period. This includes an increase in sensitivity to hypoxia, establishment of afferent nerve phenotype and axonal pruning under control of trophic factors such as BDNF and GDNF (Brady et al. 1999). These functional changes are largely under control of the rise in Pa_{O_2} which occurs at the time of birth. For instance, birthing into a low oxygen environment, which limits

*Corresponding author: David F Donnelly, Department of Pediatrics, Section of Respiratory Medicine, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06520, Tel: 203-785-4999, FAX: 203-785-6337, David.Donnelly@Yale.edu.

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the rise in Pa_{O_2} at the time of birth, delays the developmental increase in oxygen sensitivity and results in an impaired ventilatory response to acute hypoxia (Hanson et al. 1989b).

Birthing into an high oxygen environment also results in an impairment of the ventilatory response to acute hypoxia. When started in the first week or two of life and lasting 2-4w, hyperoxia ($\text{F}_{\text{I}_{\text{O}_2}}=0.6$) results in a life-long impairment of the ventilatory response to acute hypoxia, despite a prolonged (3-4mo) return to a normoxia environment (Ling et al. 1996; Ling et al. 1997; Bavis et al. 2002). It is unlikely that the effect is simply due to oxygen toxicity because it is unaffected by antioxidant supplementation (Bavis et al. 2008) and occurs at inspired oxygen levels as low as 30% O_2 as demonstrated in newborn kittens exposed to hyperoxia for 12-23 days (Hanson et al. 1989a) and newborn rats (Eden et al. 1986; Bavis et al. 2003). In contrast, similar effects are not observed when adults are treated with hyperoxia.

The effects of developmental hyperoxia are largely due to impaired peripheral chemoreceptor function as demonstrated by a reduced whole-sinus-nerve response to cyanide, hypoxia and asphyxia (Ling et al. 1997; Bisgard et al. 2003) and a major reduction in the number of glomus cells and sinus nerve axons (Erickson et al. 1998; Wang & Bisgard 2005). Because the loss of whole-nerve chemoreceptor activity could be partially or fully explained by a loss of afferent axons, we previously examined the action potential (AP) activity of single chemoreceptor axons to determine if their individual spiking rates were affected by 2-weeks of hyperoxia exposure ($\text{F}_{\text{I}_{\text{O}_2}}=0.6$) starting in the post-natal period. After 14d exposure to hyperoxia, single axonal spiking rates were greatly reduced during both normoxia and acute hypoxia. In addition, nerve conduction time was significantly lengthened, suggesting impairment of nerve excitability after this treatment period (Donnelly et al. 2005).

The present study was designed to resolve the time course of the hyperoxia-induced impairment of afferent spiking activity and to begin to address the mechanism of the impairment. Two questions are addressed: i) what is the time course of hyperoxia-induced impairment of single-unit, afferent nerve responses to acute hypoxia and ii) what is the time course of alterations, if any, in glomus cell response to acute hypoxia, in terms of both catecholamine release and increase in intracellular calcium. Glomus cells are presynaptic to the afferent nerve terminals, possess oxygen-sensitive K^+ currents and increase their calcium and secretory rates when exposed to acute hypoxia (Gonzalez et al. 1994); they are generally considered to be the site of hypoxia transduction in the carotid body. Previous data regarding hyperoxia effects on glomus cell function were obtained following a prolonged return to normoxia (Prieto-Lloret et al. 2004).

2. Methods

Experiments were undertaken with the approval of the Yale Animal Care and Use Committee and Animal Care and Use Committee of the University of Arkansas for Medical Sciences.

2.1 Animal Model

Experiments were conducted on Sprague-Dawley rat pups of both sexes. Starting on postnatal day 7 (P7), the control litters were maintained in a normoxia environment and the test litters were placed in an environmental chamber whose atmosphere was maintained at 60% oxygen (BioSpherix). Chamber CO_2 was maintained under 0.2% by a controlled leak from the chamber. Animals remained in the hyperoxia environment until studied at P8, P10, P12, P15 and P21. These ages were chosen because they were beyond the period of greatest post-natal maturation of chemoreceptor activity (Kholwadwala et al. 1992; Wasicko et al. 1999) but included ages within the post-natal window for affecting long-term alterations of chemoreceptor responsiveness (Bavis et al. 2002).

2.2 Experimental preparation for afferent nerve recording

Prior to tissue harvest, rats were deeply anesthetized by placement in a closed chamber whose atmosphere was gradually replaced with 100% CO₂. While anesthetized, the animals were removed and decapitated. Following a midline neck incision, the trachea was retracted rostrally and the carotid arteries dissected free past the carotid bifurcation. After cutting the internal and external carotid arteries and removal of the superior cervical ganglion, the vagus nerve was dissected free, centrally, past its junction with the glossopharyngeal nerve and the combined nerve was cut near its entrance to the brainstem. The ganglion was reflected over the carotid bifurcation and the tissue removed and placed in oxygenated (95% O₂/5% CO₂) saline solution (in mM: 120 NaCl, 5 KCl, 2 CaCl₂, 1 Na₂HPO₄, 1 MgSO₄, 24 NaHCO₃ and 5 glucose). In the bath, the vagus nerve and carotid arteries were dissected free from the glossopharyngeal nerve and carotid body. To aid in tissue cleaning, the complex (carotid body/sinus nerve/glossopharyngeal nerve and ganglia) was transferred to a chamber filled with saline containing collagenase (0.1%, Roche Diagnostics type P) and protease (0.02% Sigma type XIV) at 37°C for 30min with gentle agitation. The complex was further cleaned and transferred to a perfusion chamber (Warner Instruments RC-22C, chamber volume approximately 140μL) mounted on the stage of an inverted microscope equipped with Hoffman contrast optics (Zeiss Axiovert 10). The complex was superfused with oxygenated (21% O₂/5% CO₂/balance N₂) saline controlled at 37°C by an in-line heater (TC344 temperature controller; Warner Instruments). Perfusion rate was approximately 3ml/min and was limited by the flow resistance of the perfusion tubing (stainless steel, 0.030" I.D. × 5ft. Upchurch Scientific). Greater details regarding dissection and cleaning were previously given (Donnelly et al. 2000). Chamber oxygen tension was measured by phosphorescence quenching (Oxy-micro with PST-1 probe, World Precision Instruments).

Single-unit activity was recorded using a suction electrode advanced into the petrosal ganglion. Electrode tip size was approximately 30μm in diameter which allowed individual ganglion cells to enter the tip. The pipette potential was amplified with an extracellular amplifier (BAK Instruments, MDA5), passband-filtered (0.2-5kHz), displayed on an oscilloscope, digitized (10kHz sample rate) and stored on computer (Axoscope, Axon Instruments). Unit chemoreceptor activity was discriminated and timed, post-hoc, using an event detection program which identified the timing and magnitude of action potential events (CLAMPFIT9.0, Axon Instruments). The number of individual spikes per second was calculated and graphed as a function of time and fit to a 3-sec moving average for determining peak firing frequencies (Origin 7.5; Microcal Corporation).

As an aid for identification of nerve fibers projecting to the carotid body and for measurement of conduction velocity, a stimulus electrode (pipette filled with 1M NaCl; 1 MΩ impedance) was advanced into the carotid body. A constant-current stimulus (200μA)(BSI-2, BAK Instruments) was delivered at 1Hz × 0.1msec (BAK Instruments, BPG-1) and the success of the stimulus in initiating an orthodromic action potential was determined in the post-stimulus period. If an action potential was successfully evoked then the stimulus current was reduced to near threshold and about 5 sweeps stored on disk. Conduction time was measured as the time period from the start of the stimulus artifact to the time of arrival of the afferent spike at the soma. The conduction time was not translated to velocity owing to uncertainty of actual axonal length since it could not be immediately discerned where within the carotid body the action potential was initiated.

2.3 Experimental preparation for catecholamine measurement

Carotid bodies were harvested, as above, with the exception that the sinus nerve was cut and the carotid bodies harvested in isolation from the ganglion. To assess elements that are presynaptic to the afferent nerve fibers, hypoxia-induced catecholamine secretion was

measured using amperometry, as previously described (Faustino et al. 2006). Catecholamines are a major component of the dense-cored secretory granules contained in the glomus cells, and kinetics of catecholamine release have been used as an index of hypoxic responsiveness of these cells (Ortega et al. 2003). Electrodes were 5- μ m carbon fibers polypropylene insulated except for the tip (proCFE; Dagan, Minneapolis, MN). Electrodes were polarized to +200 mV against an Ag-AgCl reference electrode and advanced into the carotid body under visual observation. Current changes were acquired and digitized (Axopatch 1D and Axoscope; Axon Instruments) at a sampling rate of 10 kHz.

2.4 Experimental protocol for chemoreceptor nerve recordings and catecholamine measurements

After measurement of nerve conduction time evoked by orthodromic stimulation (nerve recording only) and baseline activity in normoxia (21% O₂/5% CO₂/bal N₂), the perfusate was switched to saline equilibrated with moderate hypoxia (12% O₂/5% CO₂/balance N₂) for a period of 2 min followed by a recovery in normoxia for 10min. After the recovery period, the perfusate was switched to saline equilibrated with severe hypoxia (0% O₂/5% CO₂/balance N₂) for a period of two minutes followed by a return to normoxia. For catecholamine measurements, after a 10min recovery period, the secretory response to 30mM K⁺ (isosmotic substitution for Na⁺) was recorded for two minutes, after which the electrode was removed from the tissue and calibrated with a solution of 2 μ M dopamine.

2.5 Experimental preparation for glomus cell isolation and measurement of intracellular calcium

These experiments were undertaken in Little Rock, Arkansas. Rat pups of the same ages as above were anesthetized with isoflurane, the preferred method of the University of Arkansas IACUC, decapitated and the carotid bifurcations removed and placed in ice-cold saline. The carotid bodies from several pups were dissected free, cut in half and placed in saline containing 1mg/ml trypsin (Sigma) and 1mg/ml collagenase (Worthington Biochemical, type 1). Cells were dispersed by gentle trituration and pelleted for 4min at 2000g. Cells were resuspended in Ham's F12 with 10% fetal calf serum and plated on coverslips coated with poly-D-lysine. Greater details are given elsewhere (Wasicko et al. 2006). Cells were loaded with the calcium-sensitive dye, FURA-2 by incubation with 4mM FURA-2 acetoxymethyl ester for 30 min at 37 °C in saline equilibrated with 21% O₂/5% CO₂/bal N₂. FURA-2 fluorescent emission was measured every 8 seconds at ~510nm in response to alternating excitation at 340 and 380nm. Images were acquired and stored using a Nikon TE300 inverted microscope and cooled CCD camera (Photometrics) under computer control (Metafluor, Universal Imaging, West Chester, PA, USA).

2.6 Experimental protocol for calcium measurements of isolated glomus cells

After loading with fura-2, the coverslip was placed in a closed microscope chamber (0.1 ml total volume) and perfused with a bicarbonate-buffered balanced salt solution (BSS) containing (mM): 118 NaCl, 23 NaHCO₃, 3 KCl, 2 KH₂PO₄, 1.2 CaCl₂, 1 MgCl₂ and 10 glucose. The saline was initially bubbled in a heated reservoir with 21% O₂/5% CO₂/bal N₂. The response to increased K⁺ was elicited by switching the perfusate for 2 min to a reservoir containing 20mM K⁺ (equimolar substitution of Na⁺). Cells were allowed to recover in normal BSS for 10 min and then challenged with hypoxia by switching the perfusate to BSS equilibrated with 5% CO₂/bal N₂ for 2min.

2.7 Data analysis

Spiking activities were calculated each second during moderate and severe hypoxic challenges and fit to a 3 second moving average. Peak spiking rates, conduction times, and peak level of

catecholamine were analyzed using two-way ANOVA with age and treatment (normoxia/hyperoxia) as grouping variables and trial-number as a covariate. If treatment had a significant effect then post-hoc testing by Student's t-test compared age-matched responses. The chance of a type I error was maintained at $P=0.05$ by modifying the critical p value, per the false discovery rate calculation of Benjamini and Hochberg (Benjamini et al. 1995).

For calcium measurements, areas of interest were selected over cells which demonstrated a rapid rise in intracellular calcium during superfusion with increased K^+ . These cells were considered as presumptive glomus cells. Results from cells in a cluster were averaged and considered as a single observation, and both single cells and clusters were included in the analysis. Intracellular calcium values ($[Ca^{2+}]_i$) were non-normally distributed and, therefore, non-parametric analysis was used. Responses to hypoxia or elevated $[K^+]_o$ at each age were compared using Kruskal-Wallis One Way Analysis of Variance on Ranks with Dunn's Method for post-hoc multiple comparisons across age. Comparisons of glomus cell $[Ca^{2+}]_i$ responses to hypoxia or elevated $[K^+]_o$ in normoxia-reared vs. hyperoxia-reared rats were made using the Mann-Whitney Rank Sum Test, with p-values adjusted for multiple comparisons.

For all statistical testing, significance was established at $P<0.05$. All results are expressed as mean $\pm$ SEM.

3. Results

Hyperoxia exposure had no significant affect on body weights over the treatment period: $19.6 \pm 1.7g$ (N=6) for P8, normoxia, $23.1 \pm 1.8g$ (N=6) for P8 (1d hyperoxia treatment), Hyperoxia; $63.8 \pm 1.7g$ (N=5) for P21, normoxia and $65.2 \pm 1.8g$ (N=5) for P21, hyperoxia (14d hyperoxia treatment). The numbers of observations for a given treatment and age are presented in the corresponding figure legends. Only one catecholamine measurement was made per carotid body. Up to 3 nerve measurements (trials) were made per carotid body with $>30min$ between measurements. Trial number, as a covariate, was not significant in any run.

3.1 Nerve responses to acute hypoxia

Baseline AP activity, as measured over 1 min time during normoxia superfusion, was not significantly different across ages for the normoxia-treated group ($P=.11$) or the hyperoxia-treated group ($P=0.44$). The mean frequency for the normoxia-treated group was 1.1 ± 0.2 Hz (N=45), which corresponds to the unit discharge frequency observed, in vivo, at $Pa_{O_2} = 85-90$ torr (Vidruk et al. 2001). In control carotid bodies, superfusion with saline equilibrated with 12% O_2 prompted a rapid rise in activity (Fig 1, 2). On average, AP activity in normoxia-treated was about 10-15 Hz and was not different across ages from P8 to P21 ($P=0.07$) (Fig 3). Corresponding, unit spiking rates were previously observed in rat carotid body, in vivo, at $Pa_{O_2} = 30-35$ torr (Vidruk et al. 2001). In contrast, AP activity for the hyperoxia-treated group changed with age ($P=0.01$). Two-way ANOVA revealed a significant interaction between age and treatment ($P=0.026$). Comparison between normoxia-treated and hyperoxia-treated groups at each age showed a significant decrease in AP activity at P12 and P15 during exposure to 12% O_2 (Fig 3A).

Superfusion with saline initially equilibrated with 0% O_2 resulted in a rapid increase in AP activity followed by a decrease (Fig 1, 2). The decrease has been noted previously and is ascribed to metabolic depression or depolarization blockade (Gonzalez et al. 1994). Age had a slight, but significant effect on the peak response to severe hypoxia in the normoxia-treated group ($P=0.04$), but post-hoc comparison could not determine which group(s) were different (Fig 3B). In the hyperoxia-treated group, age had a significant effect ($P<0.001$). Two-way ANOVA revealed significant interaction between age and treatment ($P<0.001$). Comparison between hyperoxia-treated and normoxia-treated groups at each age showed a significant

enhancement of peak nerve activity at P8 followed by a significant decrease in the peak response at P12 and beyond (Fig 3B).

3.2 Conduction time

As expected from the larger body size, age had a significant effect on conduction time for both the normoxia ($P<0.001$) and hyperoxia ($P<0.001$) groups. Two-way ANOVA revealed a significant interaction between age and treatment ($P=0.004$). At P8-P10 (1-3 days hyperoxia), conduction time was not different between hyperoxia-treated and control groups (Fig 4), but at P12 and P15 conduction time was significantly longer in the hyperoxia-treated group (Fig 4).

3.3 Hypoxia- and KCl- induced catecholamine secretion

In response to moderate (12% O_2) or severe (0% O_2) acute hypoxia, free tissue catecholamine increased in normoxia-treated carotid bodies (Fig 5). The magnitude of increase was approximately 4-times greater with 0% than with 12% O_2 (Fig 5A, 5B). In the control group, age had no significant effect on the magnitude of stimulated release for 12% O_2 ($P=0.6$), 0% O_2 ($P=0.6$) or K^+ ($P=0.8$). In the hyperoxia-treated group, age significantly affected catecholamine release for release stimulation by 0% O_2 ($P<0.001$) and K^+ ($P=0.009$) with catecholamine release decreasing with age. Age-dependent effects on catecholamine release in response to 12% O_2 were not observed in the hyperoxia group, but release values were small across all age groups (Fig 5)($P=0.2$). The interaction terms were not significant for either 12% O_2 ($P=.77$), 0% O_2 ($P=.23$) or K^+ ($P=.75$), but treatment as a main effect was significant for 12% O_2 ($P=0.003$), 0% O_2 ($P<0.001$) and K^+ ($P<0.001$). Comparisons at the same ages between normoxia-treated and hyperoxia-treated demonstrated a significant reduction in hypoxia-induced release by P14 for both moderate and severe hypoxia, although a trend was apparent earlier into the treatment period (Fig 5). Release of catecholamine caused by 30mM K^+ was also reduced at P14 and remained depressed throughout the hyperoxia treatment period (Fig 5).

3.4 Hypoxia- and KCl- induced changes in intracellular calcium

Baseline $[Ca^{2+}]_i$ levels in normoxia for the normoxia-treated control pups were (in nM) 50 ± 3 (P8, N=61), 56 ± 4 (P10, N=57), 65 ± 9 (P12, N=18), 60 ± 5 (P14, N=52) and 84 ± 6 (P21, N=36) (Fig 6). These values did not differ at any age from hyperoxia-treated pups: 67 ± 8 (P8, N=17), 47 ± 4 (P10, N=38), 77 ± 4 (P12, N=79), 50 ± 3 (P14, N=70) and 85 ± 8 (P21, N=33)(Fig 6). The mean increase in intracellular calcium ($\Delta[Ca^{2+}]_i$) in response to 20 mM KCl was not significantly different in control vs. hyperoxia-treated groups at P8, P10, P12 or P14 but was significantly lower in hyperoxia-treated vs. controls at P21 ($P<0.001$) (Fig 7). As with the increase in nerve activity, the calcium response of cells treated with hyperoxia for 1d was approximately double the control values ($P<0.001$) (Fig. 7). However, by three days in hyperoxia exposure (P10) the glomus cell $[Ca^{2+}]_i$ response to 0% O_2 had declined 85% from the P8-hyperoxia level (Fig 7B). By P12 and beyond, in the hyperoxia-treated group, the glomus cell $[Ca^{2+}]_i$ responses to 0% O_2 were significantly below the normoxia group (Fig 7).

4. Discussion

The present work examined the time course of changes in pre- and post-synaptic elements within the carotid body produced by peri-natal hyperoxia. The results demonstrate a significant enhancement of the nerve response to acute hypoxia at P8 (1d hyperoxia-treatment) and a significant depression by P12 (5d hyperoxia-treatment). These nerve changes are paralleled by changes in the glomus cell calcium response to acute hypoxia. Hyperoxia also caused a time-dependent decrease in hypoxia-induced catecholamine secretion, but the time differed from that of calcium and no initial augmentation of the acute response to hypoxia was observed.

Together these data demonstrate, for the first time, that hyperoxia affects pre-synaptic elements in the carotid body and strengthens the postulate that hypoxia transduction is dependent on calcium influx to the glomus cells.

4.1 Changes in afferent nerve activities

Afferent nerve activity underwent a biphasic change during chronic hyperoxia treatment – an initial augmentation (1d hyperoxia) followed by a profound depression. Initially, after 24 hrs of treatment, the response to acute, severe hypoxia was enhanced by almost 2-fold compared to the normoxia-treated control group. Little enhancement of the nerve response was observed during stimulation with moderate hypoxia (12% O₂), but the response to moderate hypoxia was highly variable among single units. This was likely due to variability in the depth of the sensor site from the carotid body surface which presented a variable diffusion gradient between the superfusate and sensor site. Thus, some peak responses to 12% O₂ approximated that obtained during 0% O₂ superfusion and some responses barely increased above baseline. During superfusion with severe hypoxia (0% O₂) the diffusion gradient would be expected to disappear, resulting in a maximal level of stimulation at all sensor site depths before decreasing due to anoxic depression or depolarization block.

The enhancement of activity at 1d treatment was unexpected but may have several plausible explanations. Firstly, hyperoxia exposure to immature carotid bodies appears to initiate a maturational enhancement of chemoreceptor sensitivity to hypoxia. For instance, short-term ventilation of an ewe with hyperoxia enhances the subsequent responsiveness of fetal chemoreceptors to acute hypoxia (Blanco et al. 1988). However, AP rates generally exceeded that obtained from normoxia-treated rats, regardless of age, suggesting that something more than the normal maturational process is occurring. Secondly, hyperoxia may have elicited an excitatory plasticity through increased production of reactive oxygen species (ROS). Prabhakar and colleagues demonstrated that chronic intermittent hypoxia (CIH), acting through ROS generation, results in an enhanced response to acute hypoxia in the adult, but this requires 10d of treatment to observe the effect (Pawar et al. 2008). The newborn, in contrast, demonstrates enhancement following just 1d of CIH-treatment and is sustained following continued CIH or a discontinuation of CIH (Peng et al. 2004; Pawar et al. 2008). Thus, CIH and hyperoxia treatment may share similar mechanisms since both enhance chemoreceptor activity at 1d treatment, but diverge after that time.

Following the initial enhancement of chemoreceptor responsiveness, chemoreceptor activity was significantly reduced by 5 days of hyperoxia exposure and continued to decrease following longer exposures. Comparison to other work is made difficult by variations in species, age and treatment among studies. For instance, in adult cats, exposure to pure oxygen (60-67 hrs) (Lahiri et al. 1987; Lahiri et al. 1990) or oxygen at 5-atmospheres pressure (2hrs exposure) (Torbaty et al. 1993) resulted in an impairment of the carotid body response to acute hypoxia, but the pattern was different than that of the present study: Baseline AP activity was enhanced but the amount of AP increase during exposure to acute hypoxia was less. The net result was either little change in absolute AP activity during hypoxia or even a slight increase (Mokashi et al. 1991). In contrast, the present results demonstrate a reduction in chemoreceptor AP activity in response to acute hypoxia by 5 days into the hyperoxia treatment period, which suggests that the newborn response to hyperoxia may be different than that of the mature animal.

An impairment of chemoreceptor activity has also been noted with longer hyperoxia exposures. Rats maintained at 60% oxygen for two weeks demonstrated greatly reduced AP activity, whether hyperoxia was started at P1 or P14 (Donnelly et al. 2005). Rats born and maintained in 30% oxygen for 5-10 weeks also had significantly reduced chemoreceptor AP activity during hypoxia (Eden et al. 1986); this effect may have been evident sooner, but the investigators did

not study chemoreceptor responses for rats < 5 weeks of age. Also kittens born into and maintained in 30% oxygen showed reduced pauci-fiber spiking activity below 150 torr Pa_{O_2} , but normal AP activity above 150 torr (Hanson et al. 1989a). Since these data were obtained *in vivo*, were a mixture of ages and were a combination of single and pauci-unit activities, direct comparison to the present results is problematic. In any case, there appears to be a consensus that hyperoxia treatment in the newborn period for a period greater than 2 weeks results in a significant reduction in chemoreceptor response to acute hypoxia. The present study suggests that these effects begin even earlier, arising some time after 3 days of hyperoxia exposure and being well developed (~80% maximal effect) by 5 days of hyperoxia exposure.

4.2 Changes in axonal conduction velocity

As we noted previously, the conduction time for an electrically evoked orthodromic AP to arrive at the soma was significantly prolonged in the hyperoxia-treated group (Donnelly et al. 2005). In the present study, the trend was noticeable by 3-days into the hyperoxia period and was significantly increased by 5 days or more of hyperoxia-treatment. The longer conduction time is likely due to a reduction in conduction velocity, but a clear testing of this conjecture is complicated by uncertainties of the conduction path. The carotid body diameter is about 30% of the axonal distance between the carotid body and start of the petrosal ganglion, so APs arising from different parts of the carotid body may have different conduction distances. In addition, the axonal trajectory once arriving at the ganglion is unknown, but may have a complicated trajectory as shown in some dorsal root ganglion neurons (Murayama et al. 1991). Although these factors add some degree of uncertainty regarding conduction velocity, it is relevant that body weights did not differ between groups at the same age, so body dimensions were likely the same in both groups at the same age. If so, then the conduction time measures, on average, reflect differences in conduction velocity rather than conduction distance.

4.3 Presynaptic changes

Apposed to the afferent nerve endings are glomus cells, presynaptic secretory cells which are generally regarded as being the hypoxia-sensor site (Gonzalez et al. 1994). These cells possess oxygen-sensitive potassium and calcium currents which cause an increase in intracellular calcium in glomus cells in response to ambient hypoxia. The rise in calcium leads to secretory vesicle fusion and release of various neurotransmitters, of which dopamine is an important member (Gonzalez et al. 1994). Although dopamine appears to be an inhibitory agent in most species, monitoring release of catecholamine has been used as a proxy for other neurotransmitter release (Ortega et al. 2003). In this regard we observed a significant reduction in hypoxia-evoked catecholamine release from carotid bodies harvested from hyperoxia-treated rats by P12 (5d treatment). An enhanced catecholamine release was not noted at 1d hyperoxia-treatment at a time at which the nerve response had nearly doubled and the magnitude of decrease in catecholamine release at P12 was much greater than the decrease in AP activity. This suggests a dissociation between catecholamine secretion and nerve excitation, a result previously observed under other experimental conditions (Donnelly 1996; Iturriaga et al. 1996). Although a reduction in catecholamine release may not necessarily indicate a reduction in the number of secretory granules or their release, a reduction in granules is the most likely explanation. Previously, pharmacologic inhibition of tyrosine hydroxylase with α -methyl-p-tyrosine caused a large reduction in glomus-cell secretory granules as observed using electron microscopy (Docherty & McQueen 1978).

Upstream of vesicle secretion, is an hypoxia-induced rise in intracellular calcium. This undergoes an initial enhancement at 1d and depression by 7d hyperoxia-treatment. Since the magnitude of K^+ evoked calcium change was not altered by hyperoxia-treatment until P21, it is unlikely that calcium channels were altered by hyperoxia-treatment. At present, two oxygen-

sensitive currents are believed to contribute to the membrane potential change during hypoxia – an oxygen sensitive K^+ leak current (Buckler 1997) and a BK-type oxygen sensitive, calcium-dependent K^+ current (Peers 1990). At present we have no knowledge on how either type current may be altered by hyperoxia in the newborn period. It is perhaps relevant that rats raised in hypoxia have reduced BK-type current (Wyatt et al. 1995), and mature rats exposed to chronic hypoxia have a reduction in non-BK-type K^+ current (Carpenter et al. 1998). If these currents serve to stabilize membrane potential and limit calcium excursions then an increased expression following hyperoxia may potentially explain the reduced calcium and nerve response to acute hypoxia.

In addition to the number of O_2 -sensitive or insensitive channels, the modulatory coupling between oxygen and channels may also be altered. Hypoxia sensitivity of BK channels is dependent on a heme-oxygenase, whose expression may be potentially modulated by environmental factors (Williams et al. 2004). Modulation of leak K^+ channels is dependent on cellular respiration and highly sensitive to disruptions of intracellular milieu, but the coupling mechanism is unresolved (Varas et al. 2007). Since hyperoxia treatment specifically inhibits the hypoxia response while leaving the response to K^+ intact, it may serve a useful experimental model to better resolve the critical coupling factors between hypoxia and glomus cell depolarization.

Knowing the time course of the hyperoxia-induced impairment may allow a molecular dissection by correlating expression changes with changes in organ function. For instance, the level of hypoxia-inducible transcription factor, HIF1- α is rapidly increased in hypoxia and results in an increase in TH expression within 1 hour of hypoxia exposure (Millhorn et al. 1997). Conversely, hyperoxia rapidly decreases HIF-protein levels (Hosford & Olson 2003) and, thus, may explain the rapid decrease in hypoxia-evoked catecholamine release following the start of hyperoxia. Reduced HIF-1 α expression through genetic manipulation is also associated with a reduced chemoreceptor nerve response to hypoxia (Kline et al. 2002), as does hyperoxia-treatment. Hence, the two models may share common downstream elements on both the pre- and post-synaptic sides and an elucidation of mechanism of one may be relevant to the other.

This work stems from the previous work of Mitchell and colleagues who demonstrated that hyperoxia exposure in the immediate newborn period in rats produced life-long impairments in the respiratory response to acute hypoxia, even after being returned to normoxia for a prolonged period of time (Ling et al. 1996; Ling et al. 1997; Bavis et al. 2002; Fuller et al. 2002; Bavis et al. 2003). The impairment occurs even with relatively small increases in oxygen tension, i.e., 30% F_{iO_2} (Hanson et al. 1989a). The critical period of exposure is within the first month of life, because exposures at later points in time failed to cause the same respiratory impairment (Bavis et al. 2002; Fuller et al. 2002; Bisgard et al. 2003). A reduction in the number of glomus cells and chemoafferent axons appears to be a major cause of the life-long impairment (Erickson et al. 1998; Wang & Bisgard 2005), but alterations in response properties of individual axons remains a possibility which has not been well examined.

Previously, we addressed the question of whether the remaining chemoreceptor axons responded normally to acute hypoxia following treatment with hyperoxia fixed period of two weeks (Donnelly et al. 2005). As reported in previous studies which utilized a variable or undefined treatment period, post-natal hyperoxia resulted in an impairment of the chemoreceptor response to acute hypoxia (Eden et al. 1986; Hanson et al. 1989a). The present work extends the previous work by defining the time course of the hyperoxia-induced alterations and demonstrating alterations at both pre- (glomus cell calcium and catecholamine secretion) and post-synaptic (nerve conduction time) sites. At present it is unresolved whether

these impairments are maintained following a return to a normoxic environment, but this will be a focus of future work in our laboratories.

4.4 Importance to human health

Human infants, especially preterm newborns, are often exposed to high inspired O₂ concentrations for prolonged periods (days - months) in the clinical setting (e.g., neonatal intensive care unit (NICU)). Preterm infants with chronic lung disease (bronchopulmonary dysplasia) treated for prolonged periods with supplemental O₂ therapy exhibit markedly blunted oxygen chemoreflex responses when tested later in life (Calder et al. 1994; Katz-Salamon et al. 1995). As reviewed by Gauda, an impairment in oxygen sensing may increase the risk of adverse outcome during respiratory perturbations associated with hypoxia or asphyxia (Gauda et al. 2004; Gauda et al. 2007). Although the mechanism for the impairment in these infants is not currently known, it may share common elements with the animal model used in the present study, i.e., a hyperoxia-induced impairment of peripheral chemoreceptor function. If so, then an understanding of the mechanism by which hyperoxia blunts chemoreceptor function may ultimately lead to a pharmacologic intervention to prevent the functional impairment.

In summary, hyperoxia in the newborn period initially enhances the carotid chemoreceptor neural response to acute hypoxia, but causes a significant decrease in AP activity by 5d into the hyperoxia period. Parallel changes occur in the glomus cell calcium response to acute hypoxia, suggesting causality between the calcium and nerve responses. In addition, nerve conduction time significantly increases by 5d hyperoxia-treatment, at the same time that AP activity is significantly reduced. Thus, environmental hyperoxia in the newborn period evokes pre- and post-synaptic changes which, together, greatly modify the chemoreceptor response to acute hypoxia. Since peripheral chemoreceptors respond fastest to blood gas perturbations, these alterations may have consequences on respiratory stability in early life.

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Figure 1.

Polygraphic recording of AP activity recorded from the soma of a petrosal neuron with projections to the carotid body. (A) Chemoreceptor recording from a P8 control rat raised in normoxia. Upper trace: chamber oxygen tension. Middle trace: polygraphic nerve recording. Lower: AP activity fit to a 3sec moving average. Acute hypoxia responses were elicited by switching for 2 min to saline equilibrated with 12% O₂ and with 0% O₂. (B) Chemoreceptor recording from a P8 rat initially raised in normoxia (P1-P7) and placed in hyperoxia (F_iO₂=0.6) for 1d.

Figure 2.

Polygraphic recording of AP activity recorded from the soma of a petrosal neuron with projections to the carotid body. (A) Chemoreceptor recording from a P15 control rat raised in normoxia. Upper trace: chamber oxygen tension. Middle trace: polygraphic nerve recording. Lower: AP activity fit to a 3sec moving average. Acute hypoxia responses were elicited by switching for 2 min to saline equilibrated with 12% O₂ and with 0% O₂. (B) Chemoreceptor recording from a P15 rat initially raised in normoxia (P1-P7) and placed in hyperoxia (F_IO₂=0.6) for 8d.

Figure 3.

Summary data of nerve activities for normoxia and hyperoxia-treated chemoreceptors.

Hyperoxia-treatment ($F_{iO_2}=0.6$) started at P7 and continued until tissue harvest/recording. (A). Peak AP activities during superfusion with 12% O₂. (B) Peak AP activities during superfusion with 0% O₂. Note increase in response to 0% O₂ at P8 (1d treatment) and decrease response beyond P12 (5d treatment). *= significantly different than normoxia treated. Number of observations: Normoxia-treated: P8 (N=9), P10 (N=4), P12 (N=12), P15 (N=12), P21 (N=8). Hyperoxia-treated: P8 (N=9), P10 (N=10), P12 (N=7), P15 (N=7), P21 (N=6).

Figure 4.

Conduction time was significantly increased by hyperoxia-treatment at P12 (5-day treatment). (A) Polygraphic recording from P21 normoxia-treated and hyperoxia-treated chemoreceptors. Conduction time was measured as the time from an electrical stimulus delivered to the carotid body and the arrival of the AP at the soma. (B) Mean $\pm$ SEM for normoxia-treated and hyperoxia-treated groups. Conduction time was significantly lengthened at P12 and beyond (5d hyperoxia-treatment).

Figure 5.

Catecholamine release is significantly decreased by hyperoxia exposure. Values are the peak tissue values recorded during exposure to moderate (12% O₂) or severe (0% O₂) hypoxia. (A) Peak tissue levels of catecholamine during superfusion with moderate hypoxia for two minutes. Release was significantly reduced by P12 (5d treatment) (B) Peak tissue levels of catecholamine during superfusion with severe hypoxia for two minutes. Release was significantly reduced by P12. (C) Peak tissue levels of catecholamine during superfusion with 30mM K⁺ for two minutes. *= significantly different than normoxia treated. Number of observations: Normoxia-treated: P8 (N=13), P10 (N=7), P12 (N=10), P15 (N=10), P21 (N=7). Hyperoxia-treated: P8 (N=8), P10 (N=6), P12 (N=6), P15 (N=6), P21 (N=9).

Figure 6.

Representative $[\text{Ca}^{2+}]_i$ responses from coverslips prepared from cells harvested from a normoxia-treated rat at P8 (A), hyperoxia-treated for 1d at P8 (B), normoxia-treated rat at P21 (C) and hyperoxia-treated for 14d at P21 (D). Note relative stability in the response to raised K^+ and initial enhancement of the calcium response to hypoxia at P8 and reduction at P21.

Figure 7.

Summary data of average intracellular calcium levels of normoxia and hyperoxia-treated glomus cells. Hyperoxia-treatment ($F_{O_2}=0.6$) started at P7 and continued until tissue harvest/recording. (A). Calcium response during superfusion with saline containing 20mM $[K^+]_o$. Treatment groups only differed at P21. (B) Calcium response during superfusion with saline equilibrated with 0% O_2 -5% CO_2 -bal N_2 . Hyperoxia-treatment caused an initial enhancement of the response to hypoxia at P8 (1d treatment) and a significant decrease at P8 and beyond. Values are mean $\pm$ SEM. Statistical significance established by non-parametric Dunn's Method for post-hoc multiple comparisons across age. *= significantly different than normoxia treated.