Chronic hyperoxia alters the early and late phases of the hypoxic ventilatory response in neonatal rats

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Bavis RW, Young KM, Barry KJ, Boller MR, Kim E, Klein PM, Ovrutsky AR, Rampersad DA. Chronic hyperoxia alters the early and late phases of the hypoxic ventilatory response in neonatal rats. J Appl Physiol 109: 796–803, 2010. First published June 24, 2010; doi:10.1152/japplphysiol.00510.2010.—Chronic hyperoxia during the first 1–4 postnatal weeks attenuates the hypoxic ventilatory response (HVR) subsequently measured in adult rats. Rather than focusing on this long-lasting plasticity, the present study considered the influence of hyperoxia on respiratory control during the neonatal period. Sprague-Dawley rats were born and raised in 60% O2 until studied at postnatal ages (P) of 4, 6–7, or 13–14 days. Ventilation and metabolism were measured in normoxia (21% O2) and acute hypoxia (12% O2) using head-body plethysmography and respirometry, respectively. Compared with age-matched rats raised in room air, the major findings were 1) diminished pulmonary ventilation and metabolic O2 consumption in normoxia at P4 and P6–7; 2) decreased breathing stability during normoxia; 3) attenuation of the early phase of the HVR at P6–7 and P13–14; and 4) a sustained increase in ventilation during hypoxia (vs. the normal biphasic HVR) at all ages studied. Attenuation of the early HVR likely reflects progressive impairment of peripheral arterial chemoreceptors while expression of a sustained HVR in neonates before P7 suggests that hyperoxia also induces plasticity within the central nervous system. Together, these results suggest a complex interaction between inhibitory and excitatory effects of hyperoxia on the developing respiratory control system.

METHODS

Experimental animals. Timed-pregnant Sprague-Dawley rats (SAS SD, Charles River Laboratories, Wilmington, MA) were placed into the peritoneum of pregnant rats on gestational day 17. Experimental animals were exposed to 30% O2 in normoxia or 21% O2 in hypoxia beginning at P7 and ending at P13–14. Control animals were kept on room air. Significance was determined by Student’s t-test; *P < 0.05.

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environmental chambers maintained at 60% O\textsubscript{2} approximately 1 day before giving birth; chambers were flushed with gases at sufficient flow rates to maintain <0.3% CO\textsubscript{2}. The resulting litters were raised in the chamber with their mothers for the first 14 postnatal days. Control rats were housed in the same room but were born and raised at ambient O\textsubscript{2} levels (21% O\textsubscript{2}). Individual rat pups were removed from the cage at P4, P6–7, or P13–14 for determination of ventilation or metabolism (see below) and then returned to their home cages; although rats from the same litter were studied at multiple ages, rats were marked to ensure that each individual was studied only once. Rats were maintained on a 12:12-h light-dark cycle throughout the study and provided food and water ad libitum.

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

Ventilation measurements. Ventilation was measured for neonatal rats aged P4, P6–7, and P13–14 from 10 control litters and 9 hyperoxia litters; individuals from 6–7 separate litters were used at each age per treatment group.

Ventilation measurements were made using a customized head-body plethysmograph similar to the design described by Mortola (34) but modified to include a separate head compartment for administering test gas mixtures. The plethysmograph consisted of two acrylic cylindrical chambers: a head chamber (4.75 mm ID; ~70 ml) through which test gases flowed and a body chamber (3.1 cm ID; internal volume was adjusted using modeling clay to limit the backward movement of the animal). A flexible collar made from a layer of latex film (~0.15 mm thick) and a layer of Parafilm (Pechiney Plastic Packaging, Menaha, WI) was fitted around the neck of the rat, thereby isolating the head and body compartments; a layer of petroleum jelly was applied around the rat’s neck to prevent leaks. The body chamber contained a T-type thermocouple probe (IT-18, Physitemp Instruments, Clifton, NJ) to monitor air temperature as well as ports connected to a pneumotach (MLT1L, ADInstruments, Colorado Springs, CO) and a calibration syringe. The pneumotach was connected to a differential pressure transducer (ML141, ADInstruments) to monitor respiratory airflows; the pneumotach was calibrated at the start of each experiment using a 0.5-ml injection. Respiratory airflows and chamber temperature were recorded continuously to a computer at a sampling rate of 400–1,000 Hz (PowerLab SSP and Chart 5.2 software with the spirometry extension, ADInstruments). Respiratory airflows were integrated and digitally filtered (high-pass, 0.1 Hz) to obtain respiratory volumes.

The plethysmograph was situated in a temperature-controlled incubator so that the air temperature in the body chamber could be maintained at 32–34°C. Although body temperature was not measured in this study, this ambient temperature range corresponds to the nest temperature and thermoneutral zone of neonatal rats (e.g., 29, 38). Gas flow through the head chamber was maintained at ~1,000 ml/min using gas-mixing rotameters (Matheson Tri-Gas, Montgomerryville, PA) and a mass flow monitor (G265, Qubit Systems, Kingston, ON, Canada); the high gas flow rate coupled with the low-volume head chamber enabled rapid changes in inspired O\textsubscript{2}. After being weighted and sealed into the plethysmograph, the rat was exposed to 21% O\textsubscript{2} (balance N\textsubscript{2}) for 20 min followed by hypoxia (12% O\textsubscript{2}) for 20 min. O\textsubscript{2} consumption was calculated using the average gas concentrations over the final 5 min of each exposure.

Statistical analysis. Preliminary analysis revealed no interaction between the sex of the rat and the effects of hyperoxia on baseline or hypoxic ventilation at any age (2-way ANOVA; all sex x treatment, P > 0.05, data not shown). Therefore, data for male and female rats were pooled for all statistical comparisons. Similarly, preliminary analysis revealed no effect of ambient temperature (in the narrow range of 32–34°C) on any of the variables studied (linear regression; all P > 0.05, data not shown), so ambient temperature was not included as a covariate in our statistical analyses.

Body mass as well as baseline ventilation, breathing variability, and O\textsubscript{2} consumption were compared among age and treatment groups using a two-way ANOVA and Student-Newman-Keuls (SNK) post hoc tests. Ventilation and metabolism during hypoxia were also expressed as a percentage change from baseline. The time course for changes in ventilation over the 8-min hypoxic exposure was first compared between treatment groups using two-way repeated-measures ANOVA and SNK post hoc tests separately at each age. Subsequently, the HVR was divided into early and late phases by restricting the analysis to minutes 1 and 8 of the hypoxic exposure, respectively. The early and late phases of the HVR, along with the metabolic response to hypoxia, were then compared among age and treatment groups using a two-way ANOVA and SNK post hoc tests. All statistical tests were run using SigmaStat 3.11 (SPSS, Chicago, IL), and P < 0.05 was considered significant. Values are reported as means ± SE.

RESULTS

Body mass. No obvious differences were observed in the size or behavior of rats raised in normoxia (control) or 60% O\textsubscript{2} (hyperoxia) at any of the ages studied. For the ventilation study, body mass was 9.0 ± 0.4 vs. 9.7 ± 0.3 g at P4, 12.9 ± 0.3 vs. 13.7 ± 0.4 g at P6–7, and 27.8 ± 0.8 vs. 28.6 ± 0.7 g at P13–14 for control and hyperoxia rats, respectively (see Fig. 1 for
While there was a significant increase in mass with age as expected ($P < 0.001$), exposure to hyperoxia had no effect (treatment, $P = 0.09$; treatment $\times$ age, $P = 0.99$).

**Normoxic ventilation.** The effect of hyperoxia on baseline ventilation varied depending on the age group being considered (treatment $\times$ age, $P = 0.001$) (Fig. 1). Hyperoxia rats had significantly lower ventilation at P4 ($-39\%$) and P6–7 ($-26\%$) compared with control rats (both $P < 0.001$). However, since baseline ventilation decreased with age in control rats (P6–7 and P13–14 vs. P4, both $P < 0.001$) but not in hyperoxia rats (all $P > 0.05$), there was no longer a difference in baseline ventilation between groups by P13–14 ($P = 0.14$) (Fig. 1). The reduced baseline ventilation in hyperoxia rats was mediated by lower tidal volumes at all ages (treatment, $P < 0.001$), while respiratory frequency was only lower at P4 (treatment $\times$ age, $P = 0.001$; post hoc comparison at P4, $P < 0.001$) (Fig. 1).

Variability in minute ventilation was quite high in neonatal rats (Fig. 2), with mean coefficients of variation (CV) ranging from 15 to 26% across ages and treatments. There was a significant effect of age ($P < 0.001$), with the CV for baseline ventilation being about one-third less at P13–14 than at P4 or P6–7 (both $P < 0.001$). Pooled across ages, however, the CV was $\sim 20\%$ greater in hyperoxia rats than in control rats (23 vs. 19%; treatment, $P = 0.03$); no significant interaction between treatment and age was detected for breathing variability. This increased variability did not appear to be related to the occurrence of apneas (Fig. 2A). Neither the CV for tidal volume nor the CV for breath duration (i.e., instantaneous frequency) was statistically different between treatment groups when considered separately (treatment and treatment $\times$ age, all $P > 0.05$; data not shown).

**Hypoxic ventilation.** The pattern of the HVR differed between treatment groups at each age studied (treatment $\times$ time, all $P < 0.01$), and this was apparent in both the raw data and when the data were normalized as a percentage increase from baseline (Fig. 3). At P4 and P6–7, the ventilatory response to 12% O$_2$ was biphasic in the control rats, with an initial peak in sample sizes). While there was a significant increase in mass with age as expected ($P < 0.001$), exposure to hyperoxia had no effect (treatment, $P = 0.09$; treatment $\times$ age, $P = 0.99$).
the first minute of hypoxia followed in subsequent minutes by a partial return toward baseline. The secondary decline in ventilation appeared reduced at P6–7 compared with P4, and control rats exhibited a sustained increase in ventilation at P13–14 (i.e., the HVR was no longer biphasic).

In contrast, hyperoxia rats exhibited a sustained increase in ventilation at all three ages (Fig. 3). At P4, this resulted in a greater increase in ventilation at minutes 2–8 (relative to baseline) in hyperoxia rats compared with control rats (all P ≤ 0.01). At P6–7 and P13–14, however, due to a smaller initial increase in ventilation (hyperoxia vs. control at minute 1, both P ≤ 0.01), differences in the HVR tended to be smaller between hyperoxia and control rats at minutes 2–8 (see Fig. 3, right, for statistical comparisons at each minute of hypoxia). When ventilation is expressed in raw units (Fig. 3, left), however, minute ventilation was reduced in hyperoxia rats throughout the hypoxic exposure at all ages (except minute 6 at P4). This is primarily explained by the lower baseline ventilation at P4 and P6–7 and the smaller initial increase in ventilation at P6–7 and P13–14.

To compare the effects of hyperoxia on the HVR across ages, we divided the HVR (expressed as a percentage increase from baseline) into an early phase (minute 1) and a late phase (minute 8). For the early HVR (Fig. 4, left), the effect of hyperoxia varied with age (treatment × age, P < 0.001). Specifically, the early HVR was similar between hyperoxia and control rats at P4 (P = 0.10). However, the magnitude of this initial rise in ventilation increased with age in control rats (P = 0.03 for P6–7 and P = 0.02 for P13–14 vs. P4, respectively) while it decreased with age in hyperoxia rats (P = 0.03 for P13–14 vs. P4). Consequently, the early HVR was significantly reduced at P6–7 (P = 0.02) and P13–14 (P < 0.001) compared with control rats. This effect is nearly completely explained by a smaller increase in respiratory frequency during the first minute of hypoxia in hyperoxia rats (P < 0.01 at P6–7 and P < 0.001 at P13–14).

The effect of perinatal hyperoxia also varied with age for the late phase of the HVR (treatment × age, P < 0.01) (Fig. 4, right). While the control rats exhibited a progressive increase in the magnitude of the late HVR with age (P = 0.04 for P6–7 and P < 0.001 for P13–14 vs. P4, respectively), the late HVR was similar across ages in hyperoxia rats. Consistent with their sustained increase in ventilation during acute hypoxia (see above), hyperoxia rats displayed...
a greater late HVR at P4 ($P < 0.001$) and P6–7 ($P = 0.02$) compared with control rats; no differences were observed at P13–14. Differences in the HVR between treatment groups at P4 and P6–7 reflect sustained, and consequently larger, increases in tidal volume in hyperoxia rats (treatment, $P < 0.001$) (Fig. 4).

**DISCUSSION**

This study revealed several novel effects of chronic hyperoxia on normoxic ventilation and the acute HVR of neonatal rats. The major findings were 1) diminished pulmonary ventilation and metabolic $O_2$ consumption in normoxia at the younger ages studied (i.e., P4 and P6–7); 2) increased breathing variability during normoxia; 3) attenuation of the early HVR by P6–7, but after P4; and 4) a sustained increase in ventilation during hypoxia at all ages studied (vs. the normal biphasic HVR expected at the younger ages). Together, these results suggest a complex interaction between inhibitory and excitatory effects of chronic hyperoxia on the developing respiratory control system: hyperoxia enhances the late HVR, on one hand, while progressively impairing the early, carotid body-mediated phase of the HVR on the other.

**Normoxic ventilation in hyperoxia-treated rats.** Rats exposed to 60% $O_2$ from birth displayed substantially reduced ventilation in normoxia during the first postnatal week. This effect was no longer evident at P14, which is consistent with previous studies reporting no lasting effect of perinatal hyperoxia (2 wk, 60% $O_2$) on normoxic ventilation in adult rats (3, 5). Across all ages, hyperoxia-treated rats also exhibited a more variable breathing pattern. It is not clear, however, if this
P14) are 13, 9, and 9 for control and 11, 12, and 13 for hyperoxia. † treatment groups are presented; please see the text for comparisons among age groups.

Ventilation and metabolism were measured on separate groups of rats in the present study, precluding the calculation of the convection requirement for individuals. Using the group means for ventilation and O2 consumption at each age, however, the O2 convection requirement tended to be lower in hyperoxia-treated rats than in age-matched controls (P4: 21 vs. 27, P6–7: 22 vs. 27, P13–14: 24 vs. 26). Although these ratios must be interpreted cautiously since they are based on different animals studied under different experimental conditions, this analysis suggests that metabolism may not entirely explain the reduced ventilation observed in hyperoxia-treated rats. It is possible, for example, that chronic hyperoxia diminishes the carotid body’s contribution to normoxic ventilatory drive (8, 38) by reducing the number of carotid chemosensitive neurons (22) and basal chemoreceptor activity (16). Carotid body activity remains low at P14 (16) despite normalization of normoxic ventilation. Therefore, if carotid body dysfunction does contribute to reduced normoxic ventilation in the first postnatal week (i.e., P4 and P6–7), compensatory plasticity would need to emerge by P13–14 elsewhere in the respiratory control system. Consistent with this hypothesis, normoxic ventilation spontaneously recovers in neonatal rats within a week following carotid body denervation (38).

HVR in hyperoxia-treated rats. The HVR is biphasic in neonatal rats as in other mammals (7, 20). The initial increase in ventilation reflects the rapid activation of peripheral arterial chemoreceptors, primarily the carotid body. In immature mammals, however, hypoxia also activates inhibitory mechanisms in the CNS that gradually depress ventilation (7, 39). In our study, we observed changes in both the early, carotid body-mediated phase of the HVR and the late phase of the HVR in response to chronic hyperoxia. Hyperoxia-treated and control rats had similar metabolic responses to hypoxia, so observed differences in the HVR are unlikely to involve changes in O2 demand.

The magnitude of the early phase of the HVR increased with age in control rats but decreased with age in hyperoxia-treated rats. The early HVR often increases during the neonatal period in mammals (7) and mirrors the well-established postnatal maturation of carotid body O2 sensitivity (11, 15). The decrease in the early HVR in hyperoxia-treated rats, however, likely reflects progressive impairment of carotid body function. The time course for changes in carotid body function during chronic hyperoxia is only beginning to be elucidated but correlates well with the observed changes in the early HVR. The number of carotid body glomus cells and chemosensitive neurons decreases within the first week of exposure to 60% O2 (22), and these effects may be apparent in as few as 4 days (Dmitrieff EF, Broge TA Jr, Piro SE, Bavis RW, unpublished observations). In addition, Donnelly et al. (17) studied carotid chemoreceptor single-unit activity and glomus cell calcium responses to hypoxia in rats exposed to 60% O2 beginning at P7. These experiments revealed diminished O2 sensitivity of carotid chemoreceptors after only 5 days in hyperoxia. Although additional plasticity downstream of the carotid body...
cannot be excluded, these data indicate that attenuation of the early HVR at P6–7 and P13–14 is caused by abnormal carotid body development. Indeed, carotid body dysfunction is primarily responsible for the long-lasting, if not permanent, attenuation of hypoxic responses observed in adult rats after perinatal hyperoxia (23, 32).

Control rats exhibited a biphasic ventilatory response to 12% O2 at P4 and P6–7, but not at P13–14. The observed decrease in the magnitude of ventilatory depression with advancing age is consistent with normal postnatal maturation of the HVR (7, 20). For example, Eden and Hanson (20) reported a significant ventilatory decline between minutes 1 and 4 of hypoxia in their P5 rats, but not in P7 or P14 rats, in response to 12% O2; at younger ages (P1 and P3), ventilation often fell below the baseline, normoxic value by the fourth minute of hypoxia. While the age at which the biphasic HVR transitioned into a mature, sustained increase in ventilation varied with the severity of hypoxia (i.e., the biphasic HVR was evident in older rats when lower inspired O2 levels were used), the HVR appeared fully mature by P14.

In contrast to our control rats, we did not observe a biphasic HVR in hyperoxia-treated rats at any of the ages studied; this result confirms and extends a similar observation for P5 hyperoxia-treated rats (30% O2 from birth) reported by Eden and Hanson (19) in a conference abstract. Whereas previous studies have emphasized changes in carotid body function after perinatal hyperoxia (reviewed in Ref. 1), the sustained increase in ventilation in young hyperoxia-treated rats suggests that hyperoxia is also altering central components of the HVR. Indeed, it appears as though hyperoxia is hastening maturation of the late HVR, perhaps shifting the balance from inhibitory toward excitatory neuromodulation in the CNS at an earlier age. Along these lines, it is important to note that the enhanced late HVR in hyperoxia-treated rats at P4 and P6–7 is due to a sustained increase in tidal volume (consistent with the normal maturation of the late HVR in rats; see Ref. 20) rather than an abnormally high frequency response.

Whether or not hyperoxia alters the rate or timing of normal maturational processes, it is also possible that some other form of excitatory plasticity contributes to the enhanced late HVR of hyperoxia-treated neonatal rats. Hyperoxia has been shown to have a variety of effects on respiratory control in adult animals, often mediated by reactive oxygen species (ROS) (reviewed in Ref. 14). For example, brief exposures to hyperoxia (10–15 min of 100% O2) have been shown to enhance the HVR in adult humans and rats (26, 28, 36). This plasticity is abolished by inhibitors of the O2-dependent enzyme neuronal nitric oxide synthase, implicating nitric oxide in the mechanism (26). Moreover, Mulkey et al. (35), using the brain stem slice preparation, demonstrated that hyperoxia increases the firing rate in a subpopulation of neurons in the solitary complex linked to respiratory control. Although neither vitamin E nor a powerful superoxide dismutase mimetic were effective at blocking long-lasting effects of perinatal hyperoxia on the adult carotid body and/or HVR (5), these experiments cannot exclude a role for ROS in transient plasticity in neonatal respiratory control.

O2 as a stimulus for postnatal maturation of respiratory control. In placental mammals, birth is associated with a rapid rise in arterial O2. This “relative hyperoxia” is recognized as an important stimulus for physiological changes that mark the transition from fetal to neonatal life, including alterations in circulation (12), brain neurochemistry (30), and breathing (10). Perhaps the best evidence for this in respiratory control has been heterokairy in the postnatal maturation of carotid body O2 sensitivity. Heterokairy is defined as plasticity in the timing and/or rate of physiological development (40), akin to heterochrony but occurring at the level of individuals instead of species. For example, hypoxia [mimicking partial pressures of O2 (PO2) in utero] delays resetting of carotid body O2 sensitivity at the level of the glomus cell in rats (41). It appears that O2 sensitivity will reset normally once the rat is returned to normoxia (41) or (eventually) even if maintained in hypoxia (21). Conversely, sheep ventilated with hyperoxic gas mixtures in utero exhibited increased carotid body sensitivity to hypoxia compared with sheep ventilated with gases to maintain normal fetal PO2 (9), suggesting premature resetting of O2 sensitivity.

These effects are presumably obscured during longer periods of hyperoxia in which carotid body function becomes progressively impaired through an independent mechanism (1).

The findings of the present study indicate that postnatal O2 may also regulate other aspects of respiratory control development. While other forms of plasticity cannot be excluded yet, changes in normoxic ventilation and metabolism and in the late HVR are consistent with earlier maturation of the respiratory phenotype during moderate hyperoxia (i.e., heterokairy). At least one other study supports the possibility that O2 regulates development of the biphasic HVR. Eden and Hanson (21) exposed rats to chronic hypoxia (13–15% O2) for the first 5–10 wk of life. This treatment nearly abolished the early HVR, as previously demonstrated in a variety of species (reviewed in Ref. 1). Interestingly, these rats showed a weak HVR to severe hypoxia (8% O2) that was distinctly biphasic (21); rats typically exhibit a sustained increase in ventilation to 8% O2 by 2 wk of age (20). In other words, it is possible that hypoxia delays the transition from the biphasic to sustained HVR, directly opposite of the effects proposed for hyperoxia in the present study. Taken together, these data suggest that perinatal O2 levels modulate the developmental programs for both the peripheral and central components of the respiratory control system.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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