Episodic hypoxia enhances late hypoxic ventilation in developing rat: putative role of neuronal NO synthase

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Gozal, David, and Evelyne Gozal. Episodic hypoxia enhances late hypoxic ventilation in developing rat: putative role of neuronal NO synthase. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R17–R22, 1999.—Nitric oxide (NO) is an excitatory neurotransmitter in the hypoxic ventilatory response (HVR). Furthermore, neuronal NO synthase (nNOS) activity in the developing rat correlates with the magnitude of late hypoxic ventilatory depression. To test the hypothesis that repeated short exposures to hypoxia may modify late HVR characteristics in young rats, we conducted 30-min hypoxic challenges in 2- to 3-day-old rat pups, before (Pre) and 6 h after (Post) they completed a series of eight cycles consisting of 5 min of hypoxia and 10 min of normoxia (Hyp-Norm) or normoxia throughout (Norm-Norm). In an additional group, similar challenges were performed after administration of either intraperitoneal vehicle or 25 mg/kg additional group, similar challenges were performed after administration of either intraperitoneal vehicle or 25 mg/kg (Hyp-Norm) or normoxia throughout (Norm-Norm). In an

IN MAMMALIAN SPECIES, the hypoxic ventilatory response (HVR) is the result of an elaborate interplay among multiple mechanisms that are activated on exposure to reduced inspired oxygen concentrations. Such mechanisms differ in their temporal characteristics (onset and duration), dependency on stimulus magnitude, overall effect on neuronal discharge (excitatory or inhibitory), specific effect on particular neuronal populations (tidal volume (VT) vs. frequency), and neurotransmitters and receptors that mediate their effects on ventilation. In this context, the concept of plasticity of respiratory control has emerged and increasingly gained acceptance in recent years. This notion implies that the ventilatory response characteristics to a particular stimulus may be modified by previous experiences with such stimulus, i.e., a memory effect (10). Periodic isocapnic hypoxia will induce serotonin-dependent increases of phrenic nerve-integrated output in anesthetized vagotomized rats that persist for minutes to hours after the final hypoxic exposure and have been termed long-term facilitation (LTF) (2, 11, 31). It has been postulated that serotonergic raphe neurons mediate LTF because repeated carotid body stimulation induces persistent increases in raphe neuronal activity (33) and stimulation of the raphe elicits LTF of phrenic nerve discharge (30). More recently, Turner and Mitchell (49) have shown the presence of LTF in awake adult goats when imposing a series of 10 cycles consisting of 3-min isocapnic hypoxic exposures separated by 5 min of isocapnic normoxia (49). Interestingly, with advancing stimulus cycles, the HVR was also correspondingly enhanced, suggesting that LTF did not affect hypoxic sensitivity.

When developing rat pups are exposed to hypoxia, a very transient initial minute ventilation (Ve) increase will occur and is followed by Ve reductions to levels below those measured in room air conditions (9, 34). We have previously shown that in addition to the neurotransmitters adenosine (36) and GABA (23, 24), nitric oxide (NO) originating from neuronal NO synthase (nNOS) activity plays a significant role in this central inhibitory process (19). Indeed, the late component of the biphasic HVR is highly correlated with the relative abundance of nNOS-containing neurons in critical regions mediating the hypoxic response in developing animals (15), such that reduced NO release and the resultant constrained ability to sustain Ve during hypoxia emerge as immediate consequences of the developmental pattern of NOS expression within the dorsocaudal brain stem. A substantial body of evidence indicates that NO modulates important elements of neural function such as memory formation and synaptic plasticity (43). In addition, the expression of NOS genes can be influenced by oxygen tension, such that increased nNOS expression will occur with tissue hypoxia (13, 42, 44). Thus we hypothesized that application of repeated intermittent exposures to hypoxia in developing rats could lead to upregulation of nNOS expression within neural structures underlying the ventilatory response to hypoxia and modify the stimulus-response characteristics to induce ventilatory enhancements during the late phase of HVR.

METHODS

The experimental protocols were approved by the Institutional Animal Use and Care Committee. Timed-pregnant Sprague-Dawley rats were obtained from a commercial vendor. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
breeder (Charles River), and delivery times were recorded. Only 2- to 3-day-old rat pups were studied because this is a postnatal age during which late hypoxic ventilatory depression is most prominent and nNOS expression in the dorsocaudal brain stem is lowest (15).

Protocol. In a first stage, ventilatory challenges with 10% O2-5% CO2-balance N2 lasting for 30 min were initially performed in each rat pup (Pre). Addition of 5% CO2 to the hypoxic gas aimed to maintain arterial partial pressure of CO2 levels within isocapnia (14). Gas switches were performed by rapidly bleeding the premixed gas mixture into the recording chamber. Animals were then allowed to recover with their dam in room air for at least 3 h and were then subjected to a series of eight cycles consisting of 5 min in 10% O2-5% CO2-balance N2 followed by 10 min in 5% CO2-balance room air (Hyp-Norm). Pups were returned to the litter, and 6 h later, 30-min hypoxic challenges were repeated (Post). As a control group, littermates underwent identical hypoxic challenges before and after eight cycles in which the hypoxic gas was replaced with 5% CO2-balance room air (Norm-Norm).

In a second stage, an additional group of 2- to 3-day-old rat pups underwent an identical protocol except that they received 0.2 ml of a mixture containing either vehicle (1:5 DMSO:saline) or the selective nNOS inhibitor 7-nitroindazole (7-NI, 25 mg/kg ip; Research Biochemicals International, Natick, MA) 6 h after completion of either Norm-Norm or Hyp-Norm cycles. The selected dosage of 7-NI has been shown to preferentially block nNOS-mediated responses (14).

In a third stage, grouped animals were subjected to an identical protocol as described above, but ventilatory recordings were not measured, and instead animals were euthanized with a pentobarbital sodium overdose for assessment of nNOS expression in the caudal brain stem on completion of the second 30-min hypoxic challenge. Ventilatory recordings. Respiratory measures were continuously acquired in the freely behaving, unrestrained animal placed in a previously calibrated 0.5-liter barometric chamber (Buxco Electronics, Troy, NY) using the methods described by Bartlett and Tenney (3) and Papaemperhofer (40). To minimize the effect of signal drift due to temperature and pressure changes outside the chamber, we used a reference chamber of similar size in which temperature was measured using a T-type thermocouple. Environmental temperature was maintained within 29–32°C, which corresponds to usual temperatures recorded in the dam. A calibration volume of 0.5 ml of air was repeatedly introduced into the chamber before and on completion of recordings. At least 30 min before the start of each protocol, animals were allowed to acclimate to the chamber, in which humidified air (90% relative humidity) warmed at 30°C was passed through at a rate of 2 l/min using a precision flow pump-reservoir system. Pressure changes in the chamber due to the inspiratory and expiratory temperature changes (7) were measured using a high-gain differential pressure transducer (model MP40–1, Valdyne). Analog signals were continuously digitized and analyzed online by a microcomputer software program (Buxco Electronics). A rejection algorithm was included in the breath-by-breath analysis routine and allowed for accurate rejection of motion-induced artifacts. Vr, respiratory frequency, and VE were computed and stored for subsequent offline analysis.

Immunoblot analysis. After a pentobarbital sodium overdose, the skull was rapidly opened and the brain was extracted, immediately placed on dry ice, and surgically dissected. The obex was visually identified, and a coronal section 1.5 mm caudal to 0.5 mm rostral to the obex was performed. Tissues corresponding to three to five animals were pooled and homogenized at 0°C with a tissue blender in 20 mM Tris-HCl buffer, pH 7.5, containing 2 mM EDTA, 0.5 mM EGTA, 25 µg/ml leupeptin, 25 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged for 10 min at 1,000 g at 4°C to remove cell debris. To separate soluble and particulate fractions, we performed subcellular fractionation by 1 h of centrifugation at 30,000 g at 4°C using a modification of the technique described by Lehel et al. (26). Supernatants were removed and considered representative of the nNOS-containing soluble fraction. Protein content was measured in each soluble fraction using the Bradford method (DC-Biorad protein assay; Bio-Rad, Richmond, CA), and samples were frozen at −70°C until analysis. SDS-PAGE was performed on 8% polyacrylamide gels by loading 40 µg of the soluble fraction for each postnatal age sample. In addition, 6 µg of protein from rat pituitary tumor cell line lysate (Transduction Laboratories, Lexington, KY) served as an nNOS-positive control. Proteins were transferred to nitrocellulose membranes and blocked with 5% milk in Tris-buffered saline (TBS) for 1 h. Soluble samples were incubated overnight with a monoclonal antibody to nNOS (70K; Transduction Laboratories, Lexington, KY) made in mouse to a protein fragment corresponding to amino acids 1,095–1,289 of human nNOS. Membranes were then washed with TBS-Tween and incubated for 1 h with a horseradish peroxidase-labeled goat anti-mouse IgG (1:20,000; Kirkegard and Perry Laboratories, Gaithersburg, MD). Proteins were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL) and semiquantitatively analyzed by scanning densitometry.

Data analysis. Values are reported as means ± SD unless indicated otherwise. For ventilatory challenges, early and late responses were assessed as the average of the first and last 3-min periods of each 30-min challenge, whereas baseline ventilation was defined as the average of the 3 min immediately preceding the hypoxic gas switch. Although the initial 3 min of a hypoxic challenge may not always correspond to the peak Ve response in a particular animal, they are primarily representative of the peripheral chemoreceptor-mediated Ve component, with little contamination from central sources, and were therefore selected for comparative analyses (41). Differences in ventilatory data among Pre and Post hypoxic ventilatory challenges were compared by paired Student’s t-tests. Differences between vehicle and 7-NI treatments for Hyp-Norm and Norm-Norm exposures were compared by ANOVA (2-way ANOVA for repeated measures) and the Newman-Keuls test. To normalize across film exposure times for the various Western blots, we expressed densitometry readings for each lane as percentage from corresponding control lysate lane. Unpaired t-tests were then used to compare nNOS ratiometric density readings in animals undergoing Hyp-Norm cycles vs. Norm-Norm cycles. A P value <0.05 was considered statistically significant.

RESULTS

Ventilatory measurements. A prominent biphasic response was present in all pups during Pre runs (Fig. 1). Ve increased during the early stages of hypoxia from 8.4 ± 1.4 to 11.9 ± 2.0 ml/min in control animals and decreased to 5.5 ± 0.9 ml/min at the end of the hypoxic challenge (P < 0.02). Such responses remained unaltered in Post when the rat pups were subjected to Norm-Norm (Fig. 1; n = 12). Indeed, the VE differences between early and late Ve (ΔVe early-late) in control animals were similar in Pre (6.4 ± 1.4 ml/min) and Post runs (6.8 ± 1.5 ml/min; P = not significant). However,
when Hyp-Norm cycles were applied, $\Delta V_e$ early-late were markedly attenuated in Post ($7.2 \pm 1.5$ ml/min in Pre vs. $4.5 \pm 1.1$ ml/min in Post; $n = 12$, $P < 0.002$). The attenuation of late hypoxic ventilatory depression was primarily mediated by sustained frequency response, with no significant contribution by VT ($P = \text{not significant}$).

Figure 2 shows the summary of ventilatory measurements aimed at testing the hypothesis that changes in nNOS activity elicited by repeated Hyp-Norm cycles may mediate the attenuation of late hypoxic ventilatory depression. When 7-NI was administered to 12 Norm-Norm pups, no significant differences in HVR trajectories occurred compared with vehicle (Fig. 2; $P = \text{not significant}$). However, 7-NI was associated with marked enhancements of late hypoxic ventilatory depression in 12 Hyp-Norm-treated rat pups (Fig. 2; $P < 0.001$). Indeed, $\Delta V_e$ early-late increased from $3.1 \pm 1.0$ ml/min in vehicle to $9.0 \pm 1.7$ ml/min after 7-NI ($P < 0.001$).

Immunoblots of nNOS in caudal brain stem. Western blotting of protein equivalents from the soluble fraction of five different samples derived from pooled caudal brain stem tissue corresponding to a total of 20 rat pups per treatment group revealed increased nNOS expression with Hyp-Norm exposures compared with Norm-Norm exposures (Fig. 3; $P < 0.01$).

**DISCUSSION**

The present study shows that episodic hypoxic exposure of young rat pups does not modify the early component of HVR when assessed 6 h later but markedly attenuates the magnitude of the late hypoxic ventilatory depression. Furthermore, such changes in the late HVR are abolished by pretreatment with the selective nNOS inhibitor 7-NI. In agreement with such findings, we have also found that nNOS expression within the caudal brain stem is significantly increased in Hyp-Norm-exposed pups.

Our ventilatory measurements during both early and late respiratory responses to hypoxia in Pre conditions are similar to those reported by previous investigators (9). Although potential errors in ventilatory measures could have been introduced in the absence of corrections for body temperature changes, such concerns are alleviated by the presence of the Norm-Norm control group. In addition, changes in oxygenation may induce significant alterations in oxygen consumption ($V_O_2$) (35), and because metabolic rates were not specifically measured, we cannot exclude that Hyp-Norm exposures were not associated with elevated metabolic rates compared with Norm-Norm. However, this is highly unlikely because baseline $V_e$ were similar in Pre and Post conditions. In addition, thermal conditions were similar before and after 7-NI administration, such that $V_e$ differences during hypoxia after nNOS inhibition are also unlikely to result from $V_O_2$ changes. Nonetheless, because $V_O_2$ was not measured, one cannot exclude with certainty that intraperitoneal administration of an nNOS blocker did not affect the ability to mount a metabolic response (5), although the absence of any effect on early and late HVR by 7-NI in the Norm-Norm group argues against such a contention.

To the best of our knowledge, this is the first study demonstrating long-lasting effects of episodic hypoxia on the hypoxic response properties of the respiratory
system in developing mammals. It has now become evident that LTF will occur after repeated carotid sinus nerve stimulation in anesthetized cats (12, 31) as well as anesthetized rats (21, 28). There is also evidence that LTF occurs in waking preparations, albeit to a lesser extent than during anesthesia, and that serotonin plays a critical role in LTF (2, 4, 31). On the basis of such previously described relationships between LTF and serotonin and our current findings of nNOS increased expression modulating the late hypoxic depression of developing rat pups, potential colocalization of these two neurotransmitters should be present. Indeed, serotonin and NOS were found to colocalize in 40–60% of neurons within the dorsal raphe nucleus of the rat (50, 51). In addition, the majority of cholinergic and serotonergic neurons in the pons are NOS positive, whereas the immunoreactivity is lower or undetectable in most of the serotonergic, aminergic, and cholinergic neurons in the medulla (8). Thus the ventilatory enhancements reported herein could be attributable to a mechanism such as LTF, in which serotonergic neurons modulate or receive modulatory inputs from NOS-positive cells.

An alternative mechanism that could potentially underlie the attenuation of the late hypoxic ventilatory depression in Hyp-Norm-exposed pups could represent a form of respiratory control conditioning. Indeed, Thomas and colleagues (47, 48) have shown that when perturbations are presented to neonatal rats, long-lasting changes in respiratory patterning will occur and can be readily uncovered during adulthood. More recently, similar classic inhibitory conditioning of ventilation was described for application of a hypercapnic gas mixture as the unconditioned stimulus in adult rats (37), suggesting that similar to other neural networks, the respiratory control network is amenable to marked plasticity changes when conditioning perturbations are applied. However, the experimental protocol applied herein did not follow a typical paradigm from which associative interactions between a conditioning stimulus and a nonconditioning stimulus would be expected to elicit long-lasting conditioned responses.

Pharmacological nNOS inhibition did not modify HVR in Norm-Norm-exposed rat pups, and this finding
is in close concordance with the relative paucity of nNOS-harboring neurons in brain stem regions mediating the ventilatory response to hypoxia (15). In contrast, a marked enhancement of the late hypoxic ventilatory depression occurred in Hyp-Norm-treated animals, such that \( V_e \) during the last 3 min of the 30-min hypoxic run was similar to that measured before application of the Hyp-Norm protocol (Fig. 2). This modification of the HVR in Hyp-Norm rat pups by 7-NI paralleled increases in nNOS expression within the caudal brain stem. Thus current studies lend further support to our hypothesis favoring an important role for NO derived from nNOS in sustaining ventilation during the second or late phase of the hypoxic response (15, 19).

The relative contributions of the two elements involved in our experimental paradigm, namely neural tissue hypoxia and increased peripheral chemoreceptor afferent input, remain unclear with respect to the observed increase in nNOS expression. So far, the gene for nNOS has demonstrated significant susceptibility to changes in oxygen tension, and increased nNOS gene expression will occur in central neurons even after short-lasting hypoxia (20, 29, 42). Hypoxia also induces the release of glutamate (32); activation of glutamate receptors in general, and more particularly of N-methyl-D-aspartate (NMDA) glutamate receptors in brain stem neurons, is critical in mounting a ventilatory response to hypoxia (27, 38, 46). On opening of the NMDA receptor channel, intracellular calcium elevation ensues, with concomitant activation of second messenger systems (16, 39); such intracellular calcium changes and kinase activation have been shown to play a critical role in nNOS activation (1, 45). Downstream recruitment of particular transcriptional regulatory elements during activation of the NMDA-NO pathway such as nuclear factor-κB (18) or AP-1 (17) could ultimately result in upregulation of specific spliced transcripts of the nNOS gene (25) and provide the framework for improved functional adaptations to the hypoxic stimulus.

In summary, the developing rat displays a characteristic biphasic ventilatory response to hypoxia, the late phase of which can be modified by application of episodic hypoxic exposures. Such experimental paradigms elicit significant alterations in nNOS expression within the caudal brain stem and appear to play a preponderant role in the attenuation of ventilatory depression during late HVR.

Perspectives

NOS has emerged in recent years as an important modulator of synaptic plasticity and has been implicated in memory formation and consolidation. In this context, it is possible that early life exposures to environmental stimuli that enhance or diminish nNOS expression may result in long-lasting modifications of the response characteristics of respiratory control networks. The present study opens the door to future research aiming to examine the modulation of synaptic relays, neurotransmitters, and receptor expression by well-controlled paradigms of pre- and postnatal stimulation.

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