Nitric oxide and respiratory rhythm in mammals: a new modulator of phase transition?

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Abstract

NO (nitric oxide) modulates several central pattern generators, but its role in respiratory rhythmogenesis and its mode of action on medullary respiratory neurons during normoxia are unknown. We analysed the actions of NO on the mammalian respiratory network at the system and cellular levels. Given systemically, the NO donor diethylamine NONOate increased post-inspiratory duration in vagus, phrenic and hypoglossal nerves, whereas blockade of NO generation with L-NAME (N\(^{-}\)-nitro-L-arginine methyl ester) produced the opposite response. At the cellular level, we pressure-ejected the NO donor on to respiratory neurons. NO had both inhibitory and excitatory effects on all types of respiratory neurons. Inhibitory effects involved soluble guanylate cyclase, as they were blocked with ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), whereas excitations were antagonized by uric acid and possibly mediated via peroxynitrite. Importantly, NO facilitated both GABA (\(\gamma\)-aminobutyric acid)- and NMDA (N-methyl-D-aspartate)-induced neuronal responses, but this was restricted to post- and pre-inspiratory neurons; other neuron types showed additive effects only. Our results support NO as modulator of centrally generated respiratory activity and specifically of ligand-mediated responses in respiratory neuron types involved in respiratory phase transition.

Introduction

NO (nitric oxide) is produced by NOS (nitric oxide synthase) during the conversion of L-arginine into citrulline [1]. Four isoforms of NOS have been identified as nNOS (neuronal NOS), eNOS (endothelial NOS), \(i\)NOS (inducible NOS) and mtNOS (mitochondrial NOS) [1]. NOSs and eNOSs are constitutive Ca\(^{2+}\)-dependent enzymes in neurons and vascular endothelial cells respectively. \(i\)NOS is an inducible Ca\(^{2+}\)-independent enzyme formed during immunological and inflammatory responses in macrophages or astrocytes. mtNOS is a constitutive form derived from nNOS, produces NO in mitochondria [2]. Thus NO is involved in many physiological processes in the CNS (central nervous system) including vasodilatation, neurotransmission, immunological reactions, maintenance of cellular metabolism and respiration [2–4].

NO’s main target is sGC (soluble guanylate cyclase) and activation of PKG (protein kinase G) [5,6], but NO also has sGC-independent effects via N-nitrosation of proteins or production of oxidative agents [3,4]. NO can modulate both glutamate and GABA (\(\gamma\)-aminobutyric acid) receptors and their release machinery [4,7]. Within the dorsomedial medulla, NO enhances both endogenous and AMPA (\(\alpha\)-amino-3-hydroxy-5-methylisoxazole-4-propionic acid)-evoked synaptic responses [8]. It also enhances NMDA (N-methyl-D-aspartate)-induced responses in vivo and in vitro [9,10], whereas GABA<sub>A</sub> receptor responses are either depressed, via a cGMP-dependent mechanism [11], or potentiated [12].

NO modulates rhythmic activities such as swallowing events [13], thalamocortical neurons [14] and olfactory locomotor central circuits [15]. This is probably achieved, in part, by its free diffusion through biological membranes [17,18]. NO’s role in breathing is described during hypoxia [19–23] as an excitatory factor to increase ventilation during low oxygen, but its role during normoxia remains unclear [24,25]. Indeed, in cats, NOS inhibitors in the pons prolonged inspiration, indicating that endogenous NO was important in respiratory phase termination [26]. In contrast, blockade of NOS in the dorso-medial respiratory group had no effect on breathing in rats [27], whereas NO-donors either increased respiration or caused apnoea [27,28]. Knockout mice for NOS revealed increased minute ventilation compared with wild-types [29]. This inconsistency may be explained by the global nature of the techniques applied as they will affect a large number of respiratory neurons of different types and hence yield a summative effect. Here, we focus on the effects of NO on single respiratory neuron types and compare the responses with those we observed systemically.

Materials and methods

In situ WHBP (working heart-brainstem preparation)

All experiments were in accordance with the U.K. Home Office Animals Scientific Procedures Act (1986). Juvenile rats
(30–35 days old; 90–130 g) were anaesthetized deeply with halothane, transected below the diaphragm, decerebrated precocically and cerebellotomized [30]. After anaesthesia was withdrawn, the thorax and head were perfused using a Ringer’s solution gassed with carbogen (95% CO₂, 5% O₂, 31°C, pH 7.4) and containing 10 mM dextrose, 125 mM NaCl, 24 mM NaHCO₃, 5 mM KCl, 2.5 mM CaCl₂, 1.25 mM MgSO₄, 1.25 mM KH₂PO₄ and 1.25% Ficoll. Perfusion pressure was continuously monitored. Phrenic, hypoglossal (XII) and vagal nerves activity were recorded, amplified (20k), filtered (8 Hz–3 kHz), rectified and integrated (τ = 100 ms).

Systemic drug application
L-NAME (N²-nitro-l-arginine methyl ester) (25 μM) and DEA-NO (diethylamine NONOate) (5 μM) were applied via the perfusate. Drug-induced pressure changes were corrected with rate adjustment of the perfusion pump.

Respiratory neuronal recording
Neurons were recorded extracellularly with a three-barrelled glass microelectrode containing the following permuted combinations of solutions: 3 M NaCl (cell recording; 8–17 MΩ), 125 mM NMDA, 500 mM GABA, 10 μM DEA-NO, 100 μM ODQ (1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one), 10 mM (uric acid), 0.9% saline, 0.1 M PBS and 0.25% DMSO for solvent control. Pressure (5–150 kPa) was applied to each barrel independently from PBS and 0.25% DMSO for solvent control. Pressure ejection of ODQ alone, a specific inhibitor of sGC, decreased AP/b by 18.2 ± 3.5% (P < 0.05; Figure 2). In these cells, ODQ alone was adjusted so that there was no effect on basal discharge (Figure 2). When DEA-NO was co-applied with ODQ, there was no decrease in firing rate (Figure 2) (P > 0.05), indicating that NO-mediated inhibition was sGC-dependent. In nine other neurons (two I, one L-I, three PI and five pre-I; n = 22). In contrast, ejection pressure of >50 kPa enhanced firing irrespective of neuronal type (+34 ± 5%; P < 0.05) and increased burst duration by 162 ± 47 ms (P < 0.05) (Figures 1B and 1C) (one E-aug, three I, eight PI and five pre-I; n = 15). Interestingly, only the inhibitory effect of DEA-NO was blocked by ODQ. In five neurons tested, DEA-NO decreased AP/b by -18.2 ± 3.5% (P < 0.05; Figure 2). In these cells, ODQ alone was adjusted so that there was no effect on basal discharge (Figure 2). When DEA-NO was co-applied with ODQ, there was no decrease in firing rate (Figure 2) (P > 0.05), indicating that NO-mediated inhibition was sGC-dependent. In nine other neurons (two I, one L-I, three PI and three pre-I), DEA-NO increased firing (+41.1 ± 10.1%; P < 0.05), and co-application of ODQ failed to alter this response. In 12 other neurons (two pre-I, two E, three I and five PI), application of uric acid, to scavenge peroxynitrite, increased firing by 13.2 ± 2.9% (P < 0.05) (Figure 2), whereas DEA-NO increased firing by 27.3 ± 4.9% (P < 0.05) (Figure 2). When drugs were co-applied, the number of AP/b was not increased (Figure 2). Thus DEA-NO-induced excitation of respiratory neuronal firing appears to be mediated by peroxynitrite.

Results
Systemic delivery
DEA-NO tended to increase the duration of the PI phase of the phrenic, XII and vagus nerves as well as increase the pre-I phase of XII discharge, although not significantly (Figure 1A) (n = 6; P = 0.12). In contrast, L-NAME decreased both PI phase duration in all nerves and pre-I phase duration in XII nerve (Figure 1A) (P < 0.05). The frequency of phrenic nerve activity was not altered. Responses were reversible after 15 min.

Respiratory neuronal recording
Respiratory neurons were recorded from the ventral respiratory column and were PI, I, pre-I, late-inspiratory (L-I) and expiratory/augmenting (E-aug) neurons. Micropressure ejection of drugs on to neurons never affected respiratory cycle duration measured on phrenic nerve recording.

Effects of NO donor on spontaneous rhythmic neuronal discharge
Low ejecting pressures (<50 kPa) reduced the number of AP/b in all respiratory neuron types (−20 ± 2%; P < 0.05) and the burst duration by 84 ± 21.4 ms (P < 0.05) (Figures 1B and 1C) (one E-aug, five I, three L-I, eight PI and five pre-I; n = 22). In contrast, ejection pressure of >50 kPa enhanced firing irrespective of neuronal type (+34 ± 5%; P < 0.05) and increased burst duration by 162 ± 47 ms (P < 0.05) (Figures 1B and 1C) (one E-aug, three I, two L-I, six PI and five pre-I; n = 17). These effects were not dependent upon control burst discharge frequency (P > 0.05) of the cells tested. Saline or solvent applications were without effect.

Distinct role for sGC and peroxynitrite in mediating NO effects
Pressure ejection of ODQ alone, a specific inhibitor of sGC, increased the number of AP/b (+24.5 ± 5.2%; P < 0.05) (four I, two L-I, four PI and five pre-I; n = 15). Interestingly, only the inhibitory effect of DEA-NO was blocked by ODQ. In five neurons tested, DEA-NO decreased AP/b by -18.2 ± 3.5% (P < 0.05; Figure 2). In these cells, ODQ alone was adjusted so that there was no effect on basal discharge (Figure 2). When DEA-NO was co-applied with ODQ, there was no decrease in firing rate (Figure 2) (P > 0.05), indicating that NO-mediated inhibition was sGC-dependent. In nine other neurons (two I, one L-I, three PI and three pre-I), DEA-NO increased firing (+41.1 ± 10.1%; P < 0.05), and co-application of ODQ failed to alter this response. In 12 other neurons (two pre-I, two E, three I and five PI), application of uric acid, to scavenge peroxynitrite, increased firing by 13.2 ± 2.9% (P < 0.05) (Figure 2), whereas DEA-NO increased firing by 27.3 ± 4.9% (P < 0.05) (Figure 2). When drugs were co-applied, the number of AP/b was not increased (Figure 2). Thus DEA-NO-induced excitation of respiratory neuronal firing appears to be mediated by peroxynitrite.
**Figure 1** Systemic and cellular effects of NO on respiratory network activity

(A) Systemic administration of L-NAME and DEA-NO induced opposite effects on respiratory phase duration in phrenic, hypoglossal and vagus nerves. Pre-I duration from hypoglossal increased with DEA-NO as well as PI phase on all nerves. L-NAME reduced the duration of these phases. (B) Micropressure ejection of DEA-NO on a PI neuron either decreased or increased the APs/burst according to the amount released, which was controlled by the pressure applied (1 bar = 100 kPa). (C) Percentage change in APs/burst according to pressure used (n = 39).

**Figure 2** Inhibitory and excitatory actions of NO are mediated by soluble guanylate cyclase and peroxynitrite respectively

(A) Upper traces: the DEA-NO inhibitory effect on an inspiratory neuron was blocked with ODQ. Lower traces: the excitatory effect of DEA-NO was abolished by uric acid, a peroxynitrite scavenger, but not by ODQ. (B) Group data for ODQ or uric acid interactions with DEA-NO (n = 17).
NO interacts with GABA and NMDA transmission in a unique respiratory-cell-type-specific manner

**Figure 3**

(A) Upper traces: DEA-NO potentiated both the GABA-evoked inhibitory effect and the excitatory actions of NMDA (lower traces). (B) GABA- (left-hand histogram) and NMDA- (right-hand histogram) induced activity of Pi and pre-I neurons was facilitated by co-application (Coapplic) of DEA-NO. Other neuron types did not demonstrate facilitation. PNA, phrenic nerve activity.

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**NO modulates GABA- and NMDA-induced respiratory neuron response**

**GABA-induced response**

GABA decreased neuronal firing ($-31.1 \pm 1.8\%$; $P < 0.01$; $n = 65$) (Figure 3A) and, using ejection pressures $<50$ kPa, DEA-NO reduced firing by $11.9 \pm 1.3\%$ ($P < 0.01$; $n = 65$). For the majority of I (88%; $n = 17$), E (84%; $n = 6$) and L-I (89%; $n = 9$) neurons, co-application of DEA-NO and GABA resulted in a summative effect, indicating no interaction. However, 64% of pre-I neurons ($n = 11$) and 63% of PI neurons ($n = 22$) showed a facilitatory inhibitory interaction. When both drugs were co-applied, neuronal firing decreased more than the summation of the individual responses (Figure 3B). For pre-I neurons, co-application decreased firing by $45.5 \pm 2.5\%$ compared with a summed decrease of $33.1 \pm 4.0\%$ ($P < 0.01$) (Figure 3B). In PI neurons, co-application decreased firing by $49.3 \pm 3.5\%$ which was greater than the algebraic summation of each separate drug effect ($33.7 \pm 4.4\%; P < 0.01$) (Figure 3B).

**NMDA-induced response**

For ejection pressure of $>50$ kPa, DEA-NO produced excitatory responses. Applied separately, both drugs excited all neuron types (DEA-NO, $20.3 \pm 2.2\%$, $P < 0.01$; NMDA, $39.9 \pm 2.7\%$, $P < 0.01$; $n = 34$). We found again a significant facilitatory interaction in the majority of PI (64%; $n = 11$) and pre-I (75%; $n = 4$) neurons when DEA-NO was co-applied with NMDA (Figure 3). For pre-I neurons, co-application of drugs increased firing by $63.2 \pm 9.7\%$, which was greater than the summation of the individual responses to each drug ($35.4 \pm 4.4\%; P < 0.01$) (Figure 3B). In PI neurons, co-application of drugs increased neuronal firing by $93.2 \pm 10.5\%$, an effect greater than the summation of individual responses ($54.3 \pm 7.5\%; P < 0.01$) (Figure 3B). In contrast, 78% of I and 80% of E neurons did not show facilitation of NMDA response in the presence of DEA-NO ($P > 0.05$).

**Discussion**

Exogenous NO can either excite or depress respiratory neurons firing via sGC and peroxynitrite respectively. Importantly, NO amplifies the responses to exogenously applied GABA and NMDA, but only on pre-I and PI neurons. The latter may explain the finding of enhanced pre-I and PI motor discharges in motor nerves when the NO donor was administered systemically.

**NO differentially modulates respiratory neuron firing**

Low ejection pressures, which we presume release low amounts of NO, inhibited all respiratory neuron types, whereas high ejection pressures (to release greater amounts of NO) excited all neurons. Both inhibitory and excitatory effects of NO on non-respiratory neuronal activity have been reported [4] and explained via dose-dependency [12,31],...
type of cell [32] or polarity of synaptic response [IPSeps (inhibitory postsynaptic potentials) compared with EPSPs (excitatory postsynaptic potentials)] [33]. We suggest that the type of response relates to NO concentration since: (i) for a given ejection pressure (e.g. low or high), responses were qualitatively similar irrespective of neuron type; and (ii) distinct intracellular signalling pathways are involved. The inhibitory action of NO was sGC-mediated, whereas NO-mediated excitations were sGC-independent, but antagonized by a peroxynitrite scavenger. We propose that endogenous NO concentration may depend on the NOS isoform activated, levels of buffers/degrading enzymes, blood flow and tissue oxygen levels, for example.

The finding that ODQ excites respiratory neuron firing suggests that endogenous sGC activity inhibits spontaneous discharge. This is in accordance with other studies [12,34,35]. Additionally, NO can have an excitatory effect on central rhythmic neuronal activity [24,25] and can enhance glutamate release [27,36]. Our data support a role for peroxynitrite in the excitatory effect of NO. Peroxynitrite promotes excitatory amino acids release from astrocytes [37], inhibits glutamate transporters, thereby preventing neuronal [38] and giall uptake [39], and induces hypersensitivity of the NMDA receptor [40]. However, it is acknowledged that the peroxynitrite scavenger that we used (uric acid) also scavenges hydroxyl radicals, which can also affect synaptic transmission [41].

Respiratory cell type specificity of NO modulation: possible physiological role

Interestingly, NO enhanced responses to exogenously applied NMDA and GABA on pre-I and PI neurons only. Why these neuron types specifically is unclear, but might relate to the specific NMDA/GABA receptors subunits that are sensitive to NO, differences in the sensitivity of sGC to NO [42], the rate of cGMP breakdown and/or phosphodiesterase activity.

Respiratory rhythmic activity in mammals comprises three neural phases: inspiration, post-inspiration and expiration. Pre-I and PI neurons may form the primary oscillator via reciprocal inhibitory connections [43], whereas glutamatergic and GABAergic neurotransmission is essential for respiratory rhythm generation in vivo [43] and in situ [44]. PI neurons participate in the inspiratory off-switch mechanism, whereas pre-I neurons are good candidates in controlling inspiratory on-switch [45]. We propose that the facilitatory interaction of NO with NMDA and GABA restricted to PI and pre-I neurons may have physiological relevance related to the postulated roles of these neuron types: NO may contribute to the phase transition from inspiration to post-inspiration and from expiration to inspiration. This is consistent with NO’s role in the pons [26].

Conclusions

NO’s effects within the respiratory network might depend on its bioavailability producing a dose-dependent effect on GABAergic and glutamatergic transmission. Under conditions of enhanced network activity (exercise, hypoxia), NO may assist in neuronal bursting following both heightened intracellular calcium concentrations (a prerequisite for nNOS activation [46]) and NO downloading from erythrocytes [18]. Reduced ATP during hypoxia can increase sGC sensitivity [47], thereby amplifying NO-mediated actions. As NO production increases, it may enhance synchronous bursting through actions on ionic membrane conductance such as the persistent sodium current [48]. In this context, it seems logical that among all the respiratory neuronal types, it should be the phase-transition neurons (pre-I and PI) that are specifically sensitive to NO-mediated enhancement of NMDA and GABA synaptic processes. We believe that our data are consistent with the idea that NO is part of an intrinsic mechanism that facilitates ventilation under physiological conditions of increased respiratory drive.

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References


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