Altered respiratory responses to hypoxia in mutant mice deficient in neuronal nitric oxide synthase

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1. The role of endogenous nitric oxide (NO) generated by neuronal nitric oxide synthase (NOS-1) in the control of respiration during hypoxia and hypercapnia was assessed using mutant mice deficient in NOS-1.

2. Experiments were performed on awake and anaesthetized mutant and wild-type control mice. Respiratory responses to varying levels of inspired oxygen (100, 21 and 12 % O_2) and carbon dioxide (3 and 5 % CO_2 balanced oxygen) were analysed. In awake animals, respiration was monitored by body plethysmograph along with oxygen consumption ($\dot{V}_{O_2}$), CO_2 production ($\dot{V}_{CO_2}$) and body temperature. In anaesthetized, spontaneously breathing mice, integrated efferent phrenic nerve activity was monitored as an index of neural respiration along with arterial blood pressure and blood gases. Cyclic 3′,5′-guanosine monophosphate (cGMP) levels in the brainstem were analysed by radioimmunoassay as an index of nitric oxide generation.

3. *Unanaesthetized* mutant mice exhibited greater respiratory responses during 21 and 12 % O_2 than the wild-type controls. Respiratory responses were associated with significant decreases in oxygen consumption in both groups of mice, and the magnitude of change was greater in mutant than wild-type mice. Changes in CO_2 production and body temperature, however, were comparable between both groups of mice.

4. Similar augmentation of respiratory responses during hypoxia was also observed in *anaesthetized* mutant mice. In addition, five of the fourteen mutant mice displayed periodic oscillations in respiration (brief episodes of increases in respiratory rate and tidal phrenic nerve activity) while breathing 21 and 12 % O_2, but not during 100 % O_2. The time interval between the episodes decreased by reducing inspired oxygen from 21 to 12 % O_2.

5. Changes in arterial blood pressure and arterial blood gases were comparable at any given level of inspired oxygen between both groups of mice, indicating that changes in these variables do not account for the differences in the response to hypoxia.

6. Respiratory responses to brief hyperoxia (Dejours test) and to cyanide, a potent chemoreceptor stimulant, were more pronounced in mutant mice, suggesting augmented peripheral chemoreceptor sensitivity.

7. cGMP levels were elevated in the brainstem during 21 and 12 % O_2 in wild-type but not in mutant mice, indicating decreased formation of nitric oxide in mutant mice.

8. The magnitude of respiratory responses to hypercapnia (3 and 5 % CO_2 balanced oxygen) was comparable in both groups of mice in the awake and anaesthetized conditions.

9. These observations suggest that the hypoxic responses were selectively augmented in mutant mice deficient in NOS-1. Peripheral as well as central mechanisms contributed to the altered responses to hypoxia. These results support the idea that nitric oxide generated by NOS-1 is an important physiological modulator of respiration during hypoxia.

It is being increasingly recognized that endogenously generated nitric oxide (NO) is associated with many biological functions including vasodilatation, platelet inhibition, immune responses, cell adhesion and neurotransmission (Moncada *et al.* 1991; Faraci & Brian, 1994; Jaffrey & Snyder, 1995). Recent studies also suggest that NO is involved in the control of breathing during hypoxia and hypercapnia. Previous studies by us (Prabhakar *et al.* 1993) and by others (Wang *et al.* 1993, 1994; Grimes *et al.* 1995) have documented the presence of nitric oxide synthase (NOS), the enzyme responsible for generating NO, in the carotid bodies. These peripheral sensory organs mediate the majority of the respiratory responses to hypoxia. In the chemoreceptor tissue, NOS is localized primarily in the nerve fibres innervating the glomus tissue and to some extent in the blood vessels (Prabhakar *et al.* 1993; Wang *et al.* 1993; Hohler *et al.* 1994; Grimes *et al.* 1995). Furthermore, neurons of the nucleus of solitarii tract (NTS), which integrate the afferent inputs from the carotid bodies, also contain NOS (Vincent & Kimura, 1992; Haxhiu *et al.* 1995). In addition, there is some evidence for the presence of NOS in the neurons near the ventral surface of the medulla, the purported site for central chemoreceptors responsible for increasing respiration during hypercapnia (Iadecola *et al.* 1993; Prabhakar *et al.* 1995). From these studies it is evident that NOS is present in peripheral and central neuronal structures that are associated with the control of breathing during hypoxia and hypercapnia.

Physiological studies have shown that inhibitors of NOS affect the respiratory responses to hypoxia. For example, respiratory responses to hypoxia are potentiated following systemic administration of NOS inhibitors (Gozal *et al.* 1996*b*). This potentiation appears to be due to the actions of NOS inhibitors at both the carotid bodies and at the central neurons in the NTS. Inhibitors of NOS augment the sensory discharge of the carotid bodies (Prabhakar *et al.* 1993; Wang *et al.* 1994; Chugh *et al.* 1994; Sun & Reis, 1994), suggesting that NO exerts an inhibitory influence on carotid body activity. The inhibitory actions of NO seem in part to be due to its action on type I cells as well as to dilatation of blood vessels in the carotid body resulting in improved oxygenation. Microinjections of NOS inhibitors in the NTS neurons, on the other hand, attenuate the respiratory stimulation during hypoxia (Ogawa *et al.* 1995).

In contrast to the effects on the responses to hypoxia, the effects of NOS inhibitors on respiratory response to CO_2 are less certain. Teppema *et al.* (1997) reported potentiation of the hypercapnic response after systemic NOS inhibition in cats, whereas Gozal *et al.* (1996*b*) found no effect of NOS inhibitors on the ventilatory response to CO_2 in rats. These observations, taken together, suggest that NO generated by NOS regulates respiration during hypoxia and probably during hypercapnia.

Two isoforms of NOS, neuronal (NOS-1) and endothelial (NOS-3), are constitutively expressed in many cells including neurons (Jaffrey & Snyder, 1995, for references). Under physiological conditions, these two isoforms are primarily responsible for endogenous generation of NO. Interestingly, the biological effects of NO seem to depend on the source of its production. For example, during focal cerebral ischaemia, NO generated by NOS-1 has been shown to exert a toxic effect on neurons, whereas NO produced by NOS-3 confers toxic resistance to neurons (Huang *et al.* 1994). Whether the modulatory effects of endogenously generated NO on respiratory responses to hypoxia and/or to hypercapnia also depend on the source of NO production, however, is not known.

Much of the information on the role of endogenous NO in the control of breathing has come from observations using various NOS inhibitors. However, most of the NOS inhibitors cannot distinguish between the NOS-1 and NOS-3 isoforms. Recently, Huang *et al.* (1993) developed transgenic mice with targeted disruption of the NOS-1 isoform. These investigators cloned and mapped the mouse NOS-1 gene, and disrupted its function by replacing the first exon with a neomycin-resistant gene. The mutant mice are viable and have no histopathological abnormalities in the central nervous system (Huang *et al.* 1993). These mutant mice, deficient in NOS-1, offer an excellent animal model for assessing the physiological importance of NO generated by NOS-1. In the present study, we examined the respiratory responses to hypoxia and hypercapnia in NOS-1-deficient mice and compared them with the responses from age-matched wild-type (WT) mice. Our results show that the respiratory response to hypoxia is selectively altered in mutant mice deficient in NOS-1, relative to WT mice, and this effect appears to be due to enhanced sensory drive from peripheral chemoreceptors as well as decreased NO generation in the brainstem.

METHODS

General preparation of animals

Experiments were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University and were performed on age-matched wild-type (WT) mice and NOS-1 mutant mice of either sex. The average weights of the animals were 22.6 ± 0.6 g for WT mice and 20.5 ± 0.5 g for mutant mice ($P > 0.05$, ANOVA). The NOS-1 mutant mice, a crossbreed between the 129/SV and C57BL/6 mouse strains, were developed as previously described (Huang *et al.* 1993). In the present study, hybrids of 129/SV and C57BL/6 strains of mice, which contained the NOS-1 protein, were used as WT controls. Experiments were performed on awake, unrestrained animals as well on anaesthetized mice.

Animals were anaesthetized with intraperitoneal injections of urethane (1.2 g kg^{-1}, Sigma). The choice of urethane was based on the reports that acid–base status is well maintained in experimental animals (Buelke-Sam *et al.* 1978). Supplemental doses of anaesthesia were given when corneal reflexes and responses to toe pinch persisted. In anaesthetized animals, routine surgical procedures included tracheal intubation and catheterization of abdominal aorta and inferior vena cava near the iliac region. Care

was taken that blood flow to major organs such as liver and kidney was not compromised. The aortic catheter was used to monitor arterial blood pressure and sample arterial blood for the analysis of blood gases. Systemic administration of fluids and/or drugs was accomplished through a catheter placed in the vena cava. Animals were allowed to breathe spontaneously. Core body temperature was monitored by a rectal thermistor probe and maintained at 37 ± 1 °C by a heating pad. At the end of the experiment, the animals were killed by intracardiac injection (0·1 ml) of euthanasia solution (Beuthanasia-D Special, Schering-Plough Animal Health, Kenilworth, NJ, USA).

Measurements of respiratory variables

In *unanaesthetized* animals, respiration was monitored by a whole body plethysmograph originally described by Bartlett & Tenney (1970) and modified by Thomas *et al.* (1992). Animals were placed in a Lucite chamber (600 ml volume) containing an inlet port for the administration of test gases and an outlet port connected to a vacuum sufficient to create a flow of 300 ml min^{-1} through the chamber, as measured by a rotameter. The chamber was connected to one side of a pressure transducer (Valydine MP45, Validyne Engineering Corp., Northridge, CA, USA), with a sensitivity of $\pm 2 \cdot 25$ cmH$_2$O, and to a reference chamber of equal volume. As the animal breathed, small changes in pressure were converted to a signal representing tidal volume (Bartlett & Tenney, 1970). The respiratory signals were amplified (BMA 830, CWE Inc., Ardmore, PA, USA) and recorded on a strip chart recorder (Dash 10, Astro-Med, Inc., West Warwick, RI, USA). The signals were also stored in a computer with respiratory acquisition software (BGPLOT, Cleveland, OH, USA). Oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) were determined by the open-circuit method (Schlenker & Farkas, 1995) using Beckman OM-14 and LB-2 analysers. Body temperature was measured using an implantable temperature transponder (BioMedic Data Systems, Seaford, DE, USA).

In *anaesthetized* animals, integrated efferent phrenic nerve activity was monitored as an index of central respiratory neuronal output. For this purpose, the phrenic nerve was isolated unilaterally at the level of the C3 and 4 spinal segments. The nerve was cut distally and placed on bipolar stainless steel electrodes. The electrical activity was filtered (band pass 0·3–1·0 kHz), amplified and passed through Paynter filters (time constant 100 ms; CWE, Inc.) to obtain a moving average signal.

Analysis of NOS protein by immunoblot analysis

Brain tissues were removed from anaesthetized WT and mutant mice and homogenized in buffer containing 0·1 M NaCl, 0·01 M Tris-Cl (pH 7·6), 0·001 M EDTA (pH 8·0), 1 μg ml^{-1} aprotinin, 1 μg ml^{-1} pepstatin, 1 μg ml^{-1} leupeptin and 100 μg ml^{-1} phenyl-methylsulphonyl fluoride (PMSF). After a $14\,000 \times g$ spin for 15 min at 4 °C, soluble proteins (100 μg) were separated on a non-reducing 6% SDS–PAGE gel and stained with Coomassie Blue. For Western blots, separated proteins were transferred at 40 V m^{-2} for 2 h at 20 °C to an Imobilon membrane (Millipore) using a Bio-Rad apparatus. After transfer, the membrane was blocked overnight at 4 °C in 5% BSA, 0·1% Tween 20, and 20 mM Tris-buffered saline, pH 7·6 (TBS-T). The membrane was incubated with a primary antibody directed against NOS-1 (Transduction Laboratories, Lexington, KY, USA) for 1 h at 25 °C, and washed three times in TBS-T for 5 min each. The membrane was incubated for 1 h in TBS-T plus 1% BSA with goat anti-rabbit secondary antibody conjugated with HRP, washed three times for 5 min in TBS-T, detected with an enhanced chemiluminescence (ECL) detection system (Amersham) and exposed to Kodak XAR film.

Measurements of cGMP levels by radioimmunoassay

Anaesthetized mice ($n = 9$ each of WT and mutant mice) were exposed to 100, 21 or 12% inspired oxygen for 5 min. At the end of the gas challenge, brainstems were removed and placed in 50 mM sodium acetate (pH 4·0), frozen in liquid nitrogen and kept at -80 °C until further analysis. Tissues were homogenized in 0·75 ml sodium acetate and the homogenate was centrifuged at $10\,000 \times g$ for 15 min at 4 °C. cGMP levels were determined in 200 μl of supernatant by radioimmunoassay (RIA) as previously described (Chang *et al.* 1990). Briefly, cGMP was acetylated with 10 μl triethanolamine : acetic anhydride (2 : 1), and then incubated with a primary antibody against cGMP (1 : 5000 dilution) and [^{125}I]cGMP (tracer) overnight. Bound fractions of cGMP were precipitated with ice-cold isopropanol and γ-globulin, and spun at $3000 \times g$ for 30 min. The supernatant was decanted and the pellet was dried and counted. Protein was assayed using a protein analysing kit (Bio-Rad Technologies) with bovine serum albumin (BSA) as standard. All assays were in duplicate and the values of cGMP are expressed as femtomoles per milligram of protein.

Experimental protocols

Series I: unanaesthetized mice. In the experiments involving unanaesthetized mice, all measurements were made between 09.00 and 12.30 h. Animals were placed in the plethysmograph chamber containing aspen bedding and allowed to acclimate to the environment for 60 min while room air flowed through the chamber. Subsequently, animals were challenged with varying levels of inspired O$_2$ or CO$_2$ as described below. In the first group of experiments, mice ($n = 8$ each of WT and mutant mice) were exposed to 100, 21 and 12% O$_2$ balanced nitrogen. Each gas challenge was given for 5 min. The protocols were repeated three times in each animal, with a 20 min interval between each protocol. O$_2$ consumption, CO$_2$ production and body temperature were measured at the end of each 5 min gas challenge. Respiratory responses to hyperoxic hypercapnia were determined in the second group of experiments ($n = 12$ WT, $n = 9$ mutant). Mice were allowed to breathe 100% O$_2$ for 5 min followed by 3 and 5% CO$_2$ balanced oxygen. The protocols were repeated three times, with a 20 min interval between each protocol.

Series II: anaesthetized mice. The effects of three levels of inspired oxygen (100, 21 and 12% O$_2$ balanced nitrogen) on efferent phrenic nerve activity and arterial blood pressure were determined in anaesthetized, spontaneously breathing mice ($n = 14$ each of WT and mutant mice). Baseline respiratory activity was monitored while the animals breathed 100% O$_2$. Subsequently, inspired gas was switched to 21% O$_2$ followed by 12% O$_2$. Each gas challenge was maintained for 5 min unless otherwise stated (see results with WT mice). Following 12% O$_2$, inspired air was switched back to 100% O$_2$. Gases were administered through a needle placed near the tracheal cannula and gas flow was controlled by a flow meter. Efferent phrenic nerve activity (integrated and raw action potentials) was recorded along with arterial blood pressure.

Mice have an average blood volume of 5·85 ml (100 g body weight)$^{-1}$ (Jacoby & Fox, 1984). As a consequence, the blood volume of an average mouse weighing 20 g is approximately ~1·2 ml. Due to this fact, in a given experiment, repeated withdrawal of arterial blood (200 μl per sample) was found to be lethal to the animal. Therefore, arterial blood gases were analysed at the end of each gas challenge in parallel experiments on WT ($n = 16$) and mutant ($n = 19$) mice. Arterial blood P_{O_2}, P_{CO_2}, and pH were analysed by a blood gas analyser (Laboratory Instruments).

Table 1. Changes in respiratory variables in unanaesthetized (A) and anaesthetized (B) wild-type and mutant mice during 100, 21 and 12% inspired O_2

		A. Unanaesthetized				B. Anaesthetized		
	n	100% O_2	21% O_2	12% O_2	n	100% O_2	21% O_2	12% O_2
		Respiratory rate (breaths min^{-1})				Respiratory rate (phrenic bursts min^{-1})		
Wild-type	8	211 ± 5.6	210 ± 3.9	$251 \pm 6.9*$	10	207 ± 9.1	212 ± 6.6	$165 \pm 11.7*$
Mutant	8	166 ± 2.6	$192 \pm 6.0*$	$231 \pm 10.1*$	14	172 ± 10.3	$199 \pm 7.2*$	$187 \pm 8.0*$
		V_T (μl (g BW)$^{-1}$)				Amplitude of phrenic activity (a.u.)		
Wild-type	8	1.82 ± 0.07	1.73 ± 0.06	$1.96 \pm 0.09*$	10	1.2 ± 0.1	$1.7 \pm 0.1*$	$2.0 \pm 0.2*$
Mutant	8	2.06 ± 0.07	$2.28 \pm 0.07*$	$2.43 \pm 0.09*$	14	1.4 ± 0.1	$2.3 \pm 0.2*$	$2.2 \pm 0.2*$
		$\dot{V}_E$ (ml min^{-1} (g BW)$^{-1}$)				Minute neural respiration (a.u. min^{-1})		
Wild-type	8	0.38 ± 0.02	0.36 ± 0.02	$0.50 \pm 0.03*$	10	230.7 ± 11.2	$350.3 \pm 18.4*$	$298.9 \pm 24.0*$
Mutant	8	0.34 ± 0.01	$0.44 \pm 0.02*$	$0.57 \pm 0.03*$	14	247.0 ± 20.8	$434.9 \pm 35.5*$	$396.0 \pm 37.6*$

Data are means $\pm$ S.E.M. $*P < 0.05$ within each group between control (100% O_2) and 21 or 12% O_2 (paired t test). BW, body weight.

The effects of hypercapnia on efferent phrenic activity were examined in another series of experiments ($n = 7$ each of WT and mutant mice). Baseline respiration was recorded for 5 min while the animals breathed 100% O_2. Hypercapnic challenge was induced by switching inspired gas to hyperoxic hypercapnic gas mixtures, i.e. 3% followed by 5% CO_2 balanced oxygen. Each gas challenge was maintained for 5 min. Following the gas challenge with 5% CO_2, inspired air was switched back to 100% O_2.

Series III: peripheral chemoreceptor sensitivity

The effects of brief hyperoxic challenge on respiration (Dejours, 1962) were examined on anaesthetized WT ($n = 12$) and mutant ($n = 20$) mice. Baseline respiration was recorded during 21% O_2 for 5 min. 100% O_2 was added to the inspired air for 20 s. Respiration (respiratory rate and tidal phrenic activity) was analysed for 30 s during 21% O_2 and during the last 15 s of hyperoxia. Breathing during the initial 5 s of hyperoxia was excluded for analysis because of the dead space of the tubing.

The effects of intravenous administration of NaCN (Fisher Scientific Co.) on respiration was examined in anaesthetized WT and mutant mice ($n = 7$ of each). Cyanide challenges were performed while the animals breathed 21% O_2. The doses of cyanide were 12.5, 25, 50 and 100 μg kg^{-1} in 50 μl of saline (0.9% NaCl) given in random order. The same volume of saline (50 μl) without cyanide served as controls. A 10 min interval was given between doses. Stock solutions of cyanide were prepared fresh in 0.9% saline before each experiment. Respiration was measured 1 min prior, and 1 min immediately after the injection of NaCN. In four experiments, carotid sinus nerves were identified where they join the glossopharyngeal nerves and sectioned bilaterally during the experiments. Ten minutes after sectioning the sinus nerves, respiratory responses to cyanide (100 μg kg^{-1}) were tested again.

Data analysis

The following variables were analysed in unanaesthetized mice: inspiratory tidal volume (V_T, μl), respiratory rate (RR, breaths min^{-1}), minute ventilation ($\dot{V}_E$, ml min^{-1}, inspiratory tidal volume $\times$ respiratory rate), O_2 consumption ($\dot{V}_{O_2}$, ml min^{-1}), CO_2 production

($\dot{V}_{CO_2}$, ml min^{-1}), respiratory quotient (RQ, $\dot{V}_{CO_2}/\dot{V}_{O_2}$), and body temperature (T_b). Respiratory variables (respiratory rate and tidal volume) were averaged for fifteen consecutive breaths over 5 min of inspired O_2 and CO_2 challenge. Tidal volume and minute ventilation were normalized to the body weight of the animal. Sighs or sniffs were excluded in the analysis. Metabolic variables and body temperature were measured at the end of each 5 min inspired O_2 challenge. Each data point in a given animal, for a given gas challenge, represents the average of three trials.

In anaesthetized mice, respiratory variables (respiratory rate and amplitude of the integrated phrenic nerve activity) were averaged over a 5 min period of each gas challenge. The following variables were analysed: respiratory rate (RR, number of phrenic bursts min^{-1}), amplitude of the phrenic activity (a.u., arbitrary units), and minute neural respiration (arbitrary units (a.u.) min^{-1}, respiratory rate $\times$ amplitude of tidal phrenic nerve activity).

All results are expressed as means $\pm$ S.E.M. Student's paired t tests were used to evaluate if each animal responded with significant increases in respiration during 21 and 12% O_2 compared with 100% O_2. Statistical significance of the magnitude of changes between WT and mutant mice was determined by one-way analysis of variance (ANOVA), with Tukey's or Dunn's *post hoc* test. P values less than 0.05 were considered significant.

RESULTS

Respiratory responses to changes in inspired oxygen

The effects of three levels of inspired oxygen on respiration were examined in awake and anaesthetized mice. In awake animals, in addition to respiration, metabolic variables (i.e. $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$ and RQ) and body temperature were also recorded. In anaesthetized animals, efferent phrenic nerve activity was monitored as an index of respiration along with arterial blood pressure and arterial blood gases.

Respiratory responses in awake animals

An example illustrating the effects of changing inspired oxygen on respiration is shown in Fig. 1A. As can be seen, respiration increased in response to 12% O_2 in both mice. However, the magnitude of increase, especially respiratory rate, was more pronounced in mutant mice. Average results are summarized in Table 1A. In response to 21% O_2, respiration was unaffected in WT mice. Further lowering the inspired oxygen to 12% O_2 resulted in a significant increase in minute ventilation. This increase in respiration was due to increases in respiratory rate as well as tidal volume. In contrast, mutant mice responded with significant increases in minute ventilation during 21% as well as during 12% O_2 . Increases in respiratory rate as well as tidal volume contributed to stimulation of breathing in mutant mice. Comparison between mutant and WT mice revealed that the magnitudes of increases in minute ventilation, tidal volume and respiratory rate during 21 and 12% O_2 were significantly greater in mutant than WT mice (Fig. 1B).

Changes in O_2 consumption, CO_2 production and body temperature

It has been well established that hypoxia results in a reduction in oxygen consumption as well as body temperature (Gautier, 1996). To assess whether changes in these variables have contributed to the ventilatory responses to hypoxia, we monitored O_2 consumption ($\dot{V}_{O_2}$), CO_2 production ($\dot{V}_{CO_2}$) and body temperature during the fifth minute of each inspired oxygen level in the same animals as above. The results are summarized in Table 2. Mutant mice had significantly greater oxygen consumption during 100% O_2 than WT controls. $\dot{V}_{O_2}$ decreased significantly in both groups of mice during 21 and 12% O_2, but the magnitude of decrease was significantly greater in mutant than WT mice ($P < 0.05$, ANOVA, Fig. 2). On the other hand, $\dot{V}_{CO_2}$ was unaffected by lowering the inspired oxygen to 21 and 12% O_2 in WT mice, whereas it decreased significantly in mutant mice during hypoxia (Table 2). The respiratory quotient (RQ) increased nearly to the same levels in both groups of mice during 21 and 12% O_2 ($P < 0.05$, ANOVA). Body
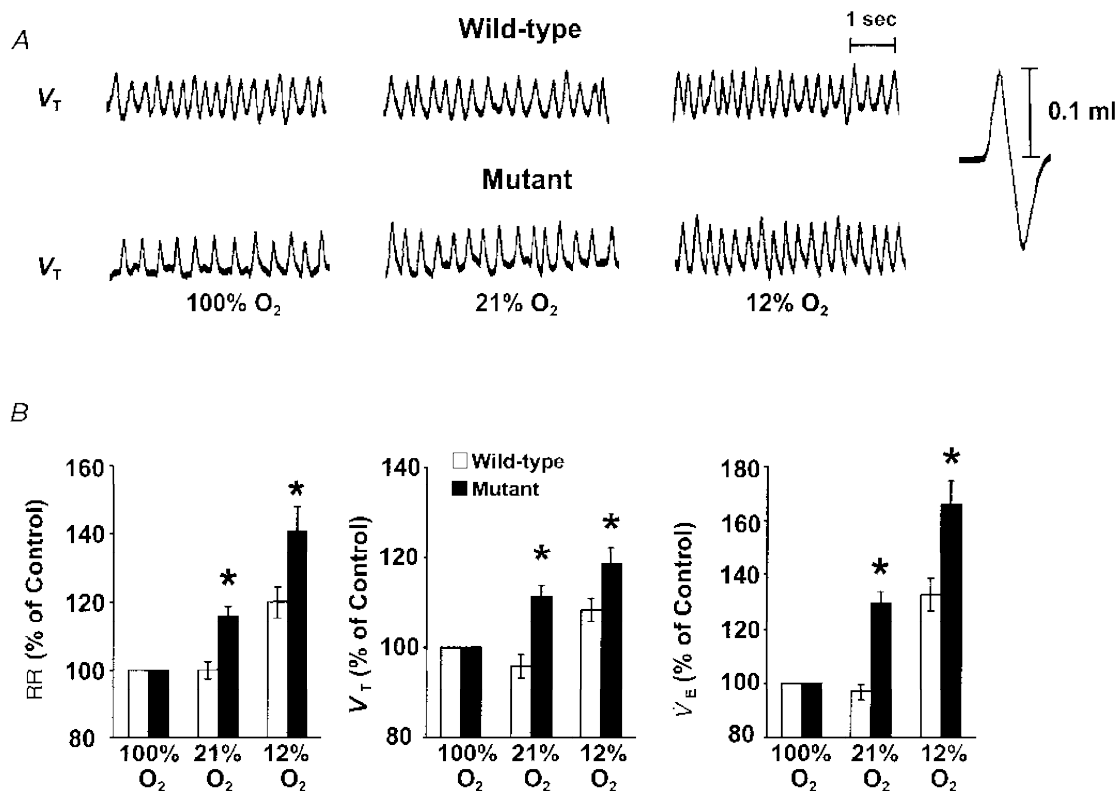

Figure 1. Respiratory responses to varying levels of inspired oxygen in unanaesthetized wild-type and mutant mice

A, representative tracing of respiratory responses to three levels of inspired oxygen in an unanaesthetized, freely moving wild-type and mutant mouse. 100% O_2, 21% O_2 and 12% O_2 indicate inspired oxygen levels. Note the greater increases in respiration in the mutant mouse during 21 and 12% O_2. *B*, comparison of respiratory responses during 21 and 12% O_2 in wild-type and mutant mice. The results are presented as a percentage of 100% O_2. Data presented as means ± s.e.m. of eight each of wild-type and mutant mice. * $P < 0.05$ (ANOVA). Note the respiratory responses (RR, V_T and $\dot{V}_E$) were greater in mutant than wild-type mice during 21 and 12% O_2.

Table 2. Changes in oxygen consumption, carbon dioxide production and body temperature in wild-type and mutant mice during 100, 21 and 12% inspired O_2

	Wild-type			Mutant		
	100 % O_2	21 % O_2	12 % O_2	100 % O_2	21 % O_2	12 % O_2
$\dot{V}_{O_2}$ (ml min^{-1})	2·30 ± 0·06	1·67 ± 0·07*	0·9 ± 0·00*	3·55 ± 0·13	1·83 ± 0·12*	0·83 ± 0·09*
$\dot{V}_{CO_2}$ (ml min^{-1})	1·28 ± 0·11	1·22 ± 0·07	1·05 ± 0·03	1·17 ± 0·06	1·29 ± 0·01	0·92 ± 0·02*
RQ	0·63 ± 0·01	0·74 ± 0·01*	1·53 ± 0·19*	0·36 ± 0·05	0·72 ± 0·05	1·21 ± 0·16*
T_b (°C)	36·95 ± 0·70	37·16 ± 0·54	36·81 ± 0·49	37·52 ± 0·38	37·48 ± 0·38	36·99 ± 0·48

Data are means ± S.E.M. from unanaesthetized mice in Table 1. * $P < 0.05$ within each group between control (100 % O_2) and 21 or 12 % O_2 (paired t test).

temperature (T_b) tended to decrease in both groups of mice during 12% O_2, but changes were not significant ($P > 0.05$, paired t test).

Respiratory responses in anaesthetized mice

In general, respiratory responses to changes in inspired oxygen in anaesthetized animals were qualitatively similar to those described in awake animals. A notable difference, however, is that WT mice showed respiratory depression during 12% O_2, whereas mutant mice tolerated hypoxia. Examples depicting the respiratory depression during hypoxia in anaesthetized WT mice, and the lack of such a response in mutant mice, are shown in Fig. 3A. Average results are summarized in Table 1B. Both groups of mice responded with significant increases in respiration during 21% O_2. In WT mice, respiratory stimulation was due to increases in tidal phrenic activity rather than increases in respiratory rate, whereas in mutant mice, increases in both the respiratory rate and the tidal phrenic activity contributed to the increases in respiration. During hypoxia, in four of the fourteen WT mice (29%) there was apnoea followed by death. Respiration could not be restored in these animals by switching inspired gas to 100% O_2. In the remaining ten animals there was a marked decrease in respiratory rate (Table 1B). In contrast, all fourteen mutant mice tolerated the hypoxic challenge and none of them died. Furthermore, their respiratory rate remained elevated during hypoxia. Comparison of the respiratory responses between both groups of mice indicated that increases in respiratory rate and neural minute respiration during 21 and 12% O_2 were significantly greater in mutant than WT mice (Fig. 3B).

Respiratory oscillations in mutant mice

In five of the fourteen anaesthetized mutant mice described above we also observed periodic episodes of increases in respiratory rate and tidal phrenic activity during 21 and 12% O_2. Each such episode of breathing lasted only a few seconds and was seen in the absence of changes in blood pressure. An example illustrating this pattern of respiratory behaviour in an anaesthetized mutant mouse is shown in Fig. 4A. As can be seen, periodic increases in respiration were absent while breathing 100% O_2. Furthermore, the time interval between the episodes decreased by reducing the inspired oxygen from 21 to 12% O_2 (Fig. 4B). These episodes of respiratory behaviour were excluded in the analysis of respiratory rate and tidal phrenic nerve activities described above. On the other hand, WT mice did
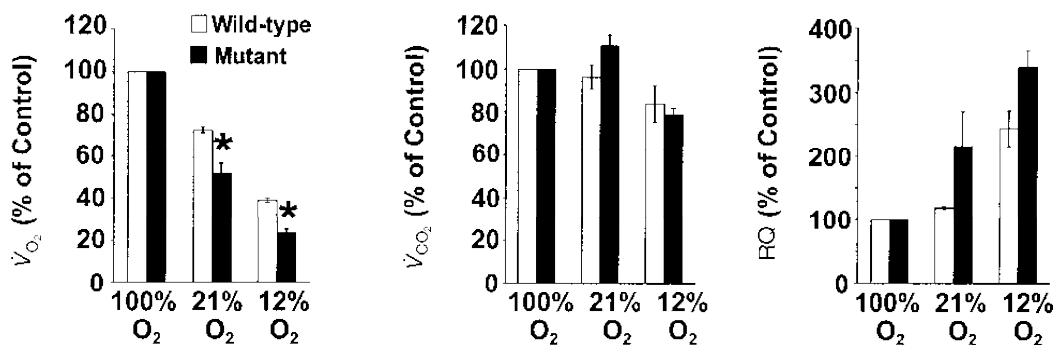

Figure 2. Comparison of changes in O_2 consumption ($\dot{V}_{O_2}$), CO_2 production ($\dot{V}_{CO_2}$) and respiratory quotient (RQ) during 21 and 12% O_2 in unanaesthetized wild-type and mutant mice

The results are presented as a percentage of 100% O_2. These data are analysed from the same mice as in Fig. 1 and presented as means ± S.E.M. * $P < 0.05$ (ANOVA). Note that the decrease in $\dot{V}_{O_2}$ was significantly greater in mutant than wild-type mice during 21 and 12% O_2.

not exhibit this pattern of respiratory behaviour either during 21 or 12% O_2. In awake mice, occurrence of sniffs precluded systematic analysis of such a breathing pattern.

Changes in arterial blood pressure and blood gases

The changes in arterial blood pressure were analysed during three levels of inspired oxygen in both groups of mice and the results are summarized in Table 3A. Basal arterial blood pressure was comparable in both groups of mice (100% O_2 WT *vs.* mutant mice, $P > 0.05$, ANOVA). Arterial blood pressure decreased in both groups of mice during 21 and 12% O_2, but the magnitude of the change was not significantly different ($P > 0.05$, ANOVA). As can be seen from Table 3B, at any given level of inspired oxygen,

changes in arterial P_{O_2} and P_{CO_2} were comparable between both groups of animals. One exception is that arterial pH during 100% O_2 was 0.064 units lower in mutant than WT mice.

Peripheral chemoreceptor sensitivity in mutant and WT mice

The results described thus far indicate that mutant mice exhibit enhanced respiratory responses to hypoxia. As NO is inhibitory to the carotid body activity (Prabhakar *et al.* 1993), the altered responses during hypoxia in mutant mice may in part be due to changes in peripheral chemoreceptor sensitivity. In order to assess this possibility the following experiments were performed on anaesthetized mice.
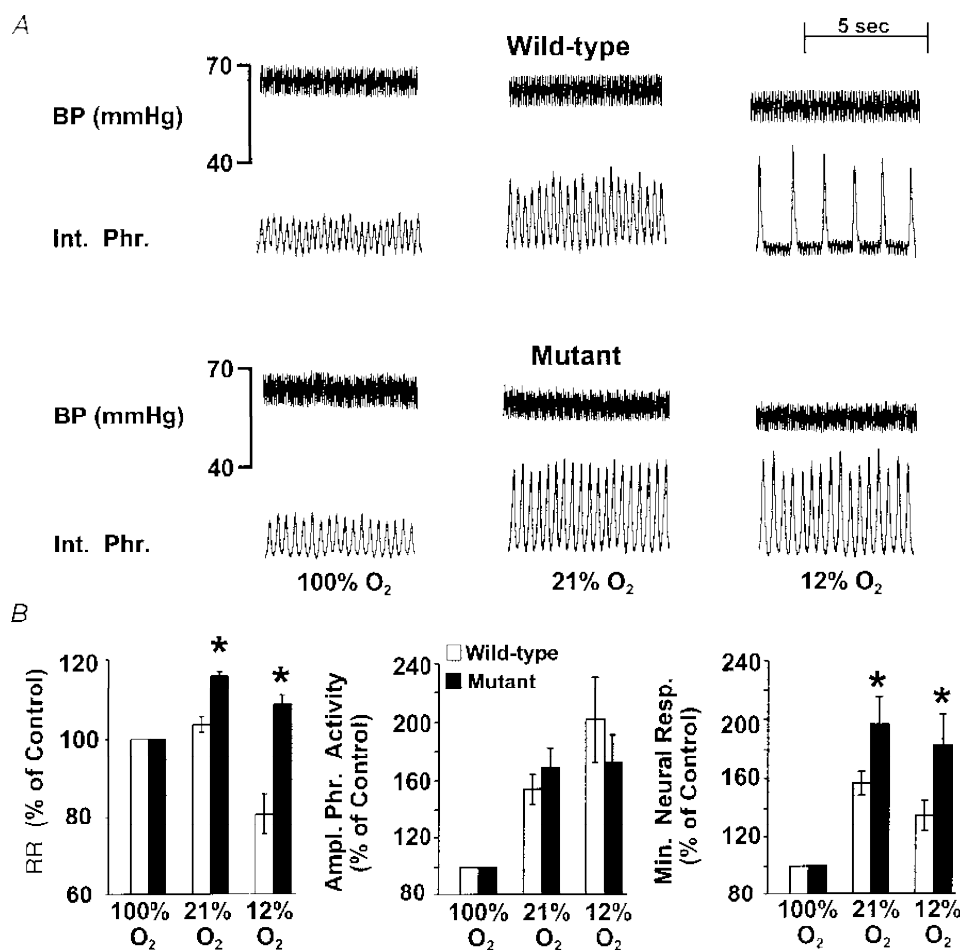

Figure 3. Respiratory responses to varying levels of inspired oxygen in anaesthetized wild-type and mutant mice

A, representative tracing of an experiment illustrating respiratory responses during 100, 21 and 12% inspired oxygen in an anaesthetized wild-type and a mutant mouse deficient in NOS-1. BP (mmHg), arterial blood pressure; Int. Phr., integrated efferent phrenic nerve activity. 100% O_2, 21% O_2 and 12% O_2 indicate inspired oxygen levels. Respiration (respiratory rate and amplitude of tidal phrenic activity) increased in response to 21% O_2 in both mice. Respiration was depressed in the WT mouse during 12% O_2, whereas it remained elevated in the mutant mouse. *B*, comparison of respiratory responses during 21 and 12% O_2 in anaesthetized wild-type and mutant mice. The results are presented as a percentage of 100% O_2. Data presented as means ± s.e.m. of wild-type ($n = 10$) and mutant ($n = 14$) mice. * $P < 0.05$ (ANOVA). Note the respiratory rate (RR) and minute neural respiration (Min. Neural. Resp.) were greater in mutant than wild-type mice during 21 and 12% O_2. Ampl. Phr. Activity, amplitude of phrenic activity.

Effects of brief hyperoxia on respiration

It has been well documented that brief inhalation of 100%
O_2 (~30 s) decreases respiration, especially the respiratory
rate (Dejours, 1962). This reduction in respiration elicited
by brief hyperoxia has been attributed to decreased
chemosensory drive from the carotid bodies (Dejours, 1962).
Therefore, the magnitude of respiratory depression during
hyperoxia is commonly used as an index of peripheral chemo-
receptor sensitivity. If carotid body sensitivity is altered in
NOS-1-deficient mice, it should be reflected in the magnitude
of decrease in the respiratory rate during hyperoxia. To test
this possibility, we compared the respiratory responses to
brief hyperoxia in mutant and WT mice; 100% O_2 was
added to the inspired air for 20 s while the animals breathed
room air. As shown in Fig. 5A, hyperoxia resulted in a
prompt reduction in respiratory rate in both mice. However,
the decrease was more pronounced in mutant mice. Average
results showed that respiratory rate decreased by $15 \pm 1\%$
in mutant mice, whereas in WT mice it was $11 \pm 2\%$ (WT
$vs.$ mutant mice; $P < 0.05$; ANOVA, Fig. 5B). Changes in
the tidal phrenic nerve activity and minute neural

respiration, however, were not significant during brief
hyperoxia in both groups of mice ($P > 0.05$, ANOVA).

Effects of sodium cyanide on respiration

In another series of experiments, we analysed the
respiratory responses to different doses of sodium cyanide
(NaCN), a potent stimulant of the carotid body, in mutant
and WT mice. As shown in Fig. 6A, cyanide-induced
respiratory stimulation was more pronounced in the mutant
mouse. Average results showed that respiration was
stimulated by cyanide in both groups of mice in a dose-
dependent manner. Administration of the same volume of
saline (i.e. vehicle), however, had no effect on respiration.
Bilateral sectioning of the sinus nerves abolished the
respiratory responses to NaCN (100 μg kg^{-1}; $n = 4$), as well
as to hypoxia, implying that the carotid bodies are
responsible for respiratory stimulation induced by cyanide
and hypoxia. Analysis of the respiratory response to
cyanide showed that increases in respiratory rate and neural
minute respiration resulting from 50 and 100 μg kg^{-1}
cyanide were significantly greater in mutant than WT mice
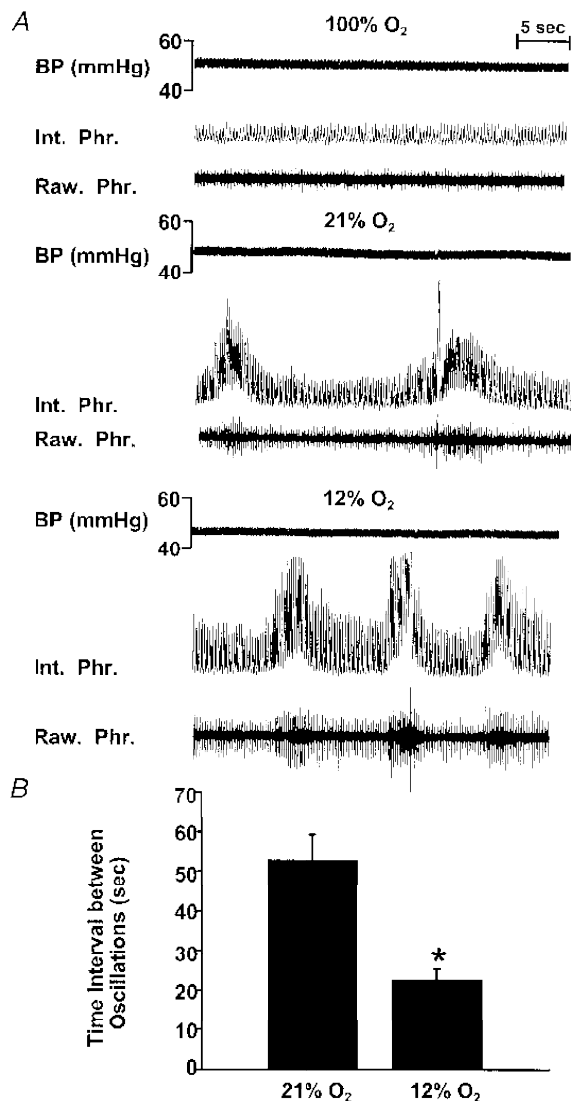($P < 0.05$; ANOVA, Fig. 6B).

Figure 4. Respiratory oscillations in mutant mice

A, representative tracing of an experiment illustrating the
periodic increases in respiratory rate and tidal phrenic
activity during inspired 21 and 12% O_2 in an anaesthetized,
spontaneously breathing NOS-1-deficient mouse. BP
(mmHg), arterial blood pressure; Int. Phr., integrated
phrenic nerve activity; Raw Phr., action potentials recorded
from the efferent phrenic nerve. 100% O_2, 21% O_2 and 12%
O_2 indicate inspired oxygen levels. Note the periodic
increases in respiratory rate during 21 and 12% O_2 breathing
but not during 100% O_2. Because of the marked increases in
respiratory rate, the integrator was not able to reset back to
baseline. The oscillatory changes in respiration could be seen
in the absence of oscillations in blood pressure. B, average
data of the time interval between oscillations during two
levels of inspired oxygen. Data presented are means $\pm$ s.e.m.
from 5 mutant mice. * $P < 0.05$ (paired t test).

Table 3. Changes in arterial blood pressure and arterial blood gases during 100, 21 and 12% inspired O_2 in anaesthetized wild-type and mutant mice

	Wild-type			Mutant		
	100% O_2	21% O_2	12% O_2	100% O_2	21% O_2	12% O_2
	A. Arterial blood pressure					
BP (mmHg)	42.4 ± 3.4	38.9 ± 3.3	33.1 ± 2.8	49.5 ± 5.3	43.8 ± 2.2	35.8 ± 2.4
n	10	10	10	14	14	14
	B. Arterial blood gas composition					
P_{O_2} (mmHg)	237.0 ± 23.4	106.3 ± 6.3	73.7 ± 2.3	241.7 ± 24.4	107 ± 3.2	75.0 ± 7.1
P_{CO_2} (mmHg)	23.1 ± 1.8	28.7 ± 1.8	24.5 ± 3.1	27.1 ± 1.7	26.2 ± 3.4	23.2 ± 1.9
pH	7.313 ± 0.02	7.27 ± 0.07	7.323 ± 0.02	7.249 ± 0.01 *	7.38 ± 0.15	7.292 ± 0.02
n	6	5	5	5	9	5

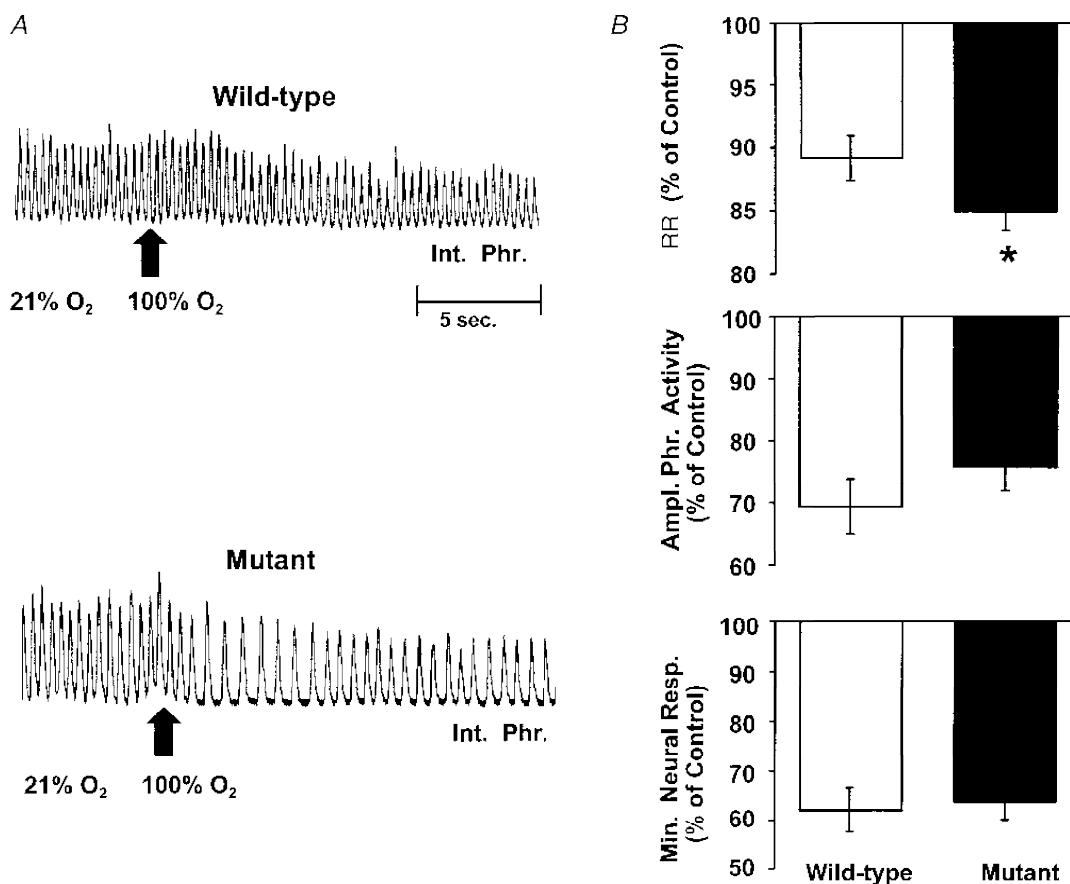Data are means $\pm$ S.E.M. * $P < 0.05$ between wild-type and mutant mice (ANOVA).

Figure 5. Respiratory responses to brief hyperoxia in anaesthetized wild-type and mutant mice
A, representative tracing of an experiment illustrating the effect of brief hyperoxia (Dejours test) on efferent phrenic nerve activity in an anaesthetized, spontaneously breathing wild-type and mutant mouse. Int. Phr., integrated efferent phrenic nerve activity. Animals breathed room air. At arrow, 100% O_2 was added to the inspired air. 100% O_2 caused prompt decreases in respiration in both types of mice, but the response was more pronounced in mutant mice. *B*, average data for changes in respiratory responses. Respiratory variables were analysed during the last 15 s of hyperoxia (for details see text). Changes in respiratory variables are presented as a percentage of 21% O_2 controls. Data presented are means $\pm$ S.E.M. from 12 wild-type and 20 mutant mice. * $P < 0.05$ (ANOVA). Note the greater decreases in respiratory rate by hyperoxia in NOS-1-deficient mice.

Analysis of cGMP levels and NOS-1 protein in the brainstem

Previous studies have indicated that NO modulates breathing during hypoxia by acting at brainstem neurons (Ogawa *et al.* 1995). The following experiments were performed to assess whether the augmented respiratory response to hypoxia seen in mutant mice was due to alterations in NO production in brainstem. It has been well established that NO increases cGMP levels by activating the enzyme soluble guanalyl cyclase (Bredt & Snyder, 1989). Given that NOS is present in the brainstem neurons, we reasoned that changes in cGMP levels should reflect NO generation in the brainstem. Therefore, we monitored cGMP

levels in brainstem extracts as an index of endogenous NO production. Brainstems were removed from anaesthetized WT and mutant mice exposed to three levels of inspired oxygen for 5 min and cGMP levels were analysed in tissue extracts by radioimmunoassay as described in Methods. In WT mice there was a progressive increase in cGMP levels on reducing inspired oxygen to 21 and 12 % O_2 (compared with 100 % O_2, $P < 0.05$; paired t test, Fig. 7A). In contrast, in mutant mice, cGMP levels remained fairly constant during 21 and 12 % O_2 (compared with 100 % O_2, $P > 0.05$, paired t test). Western blot assay revealed no detectable NOS-1 protein in the brainstem extracts from mutant mice, whereas it could readily be detected in WT mice (Fig. 7B).
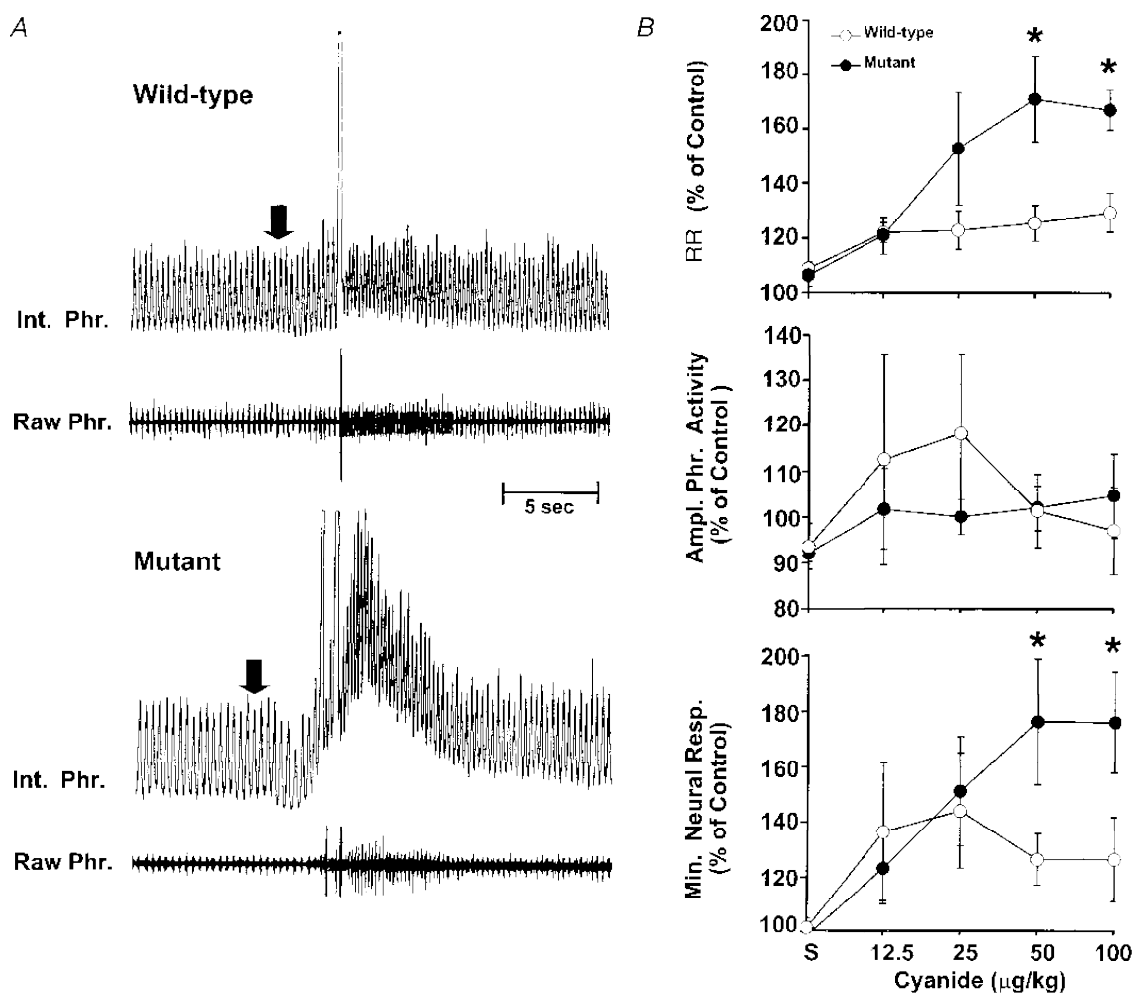

Figure 6. Respiratory responses to sodium cyanide in anaesthetized wild-type and mutant mice

A, representative tracing of an experiment illustrating the effect of systemic administration of sodium cyanide on efferent phrenic nerve activity in anaesthetized, spontaneously breathing wild-type and NOS-1 deficient mice. Cyanide (50 μg kg^{-1} i.v.) was injected at the arrow. Raw Phr., action potentials recorded from efferent phrenic nerve activity; Int. Phr., integrated phrenic nerve activity. NaCN caused prompt increases in respiration in both mice, but the response was more pronounced in mutant mice. Because of the marked increases in respiratory rate, the integrator was not able to reset back to baseline. *B*, average data for changes in respiratory variables. Results are presented as a percentage of pre-injection controls. 'S' denotes respiratory changes after saline (vehicle) injection. Data presented as means ± s.e.m. from 7 animals in each group. * $P < 0.05$ (ANOVA). Note the enhanced responses in respiratory rate and neural minute respiration at 50 and 100 μg kg^{-1} cyanide in mutant mice.

Table 4. Changes in respiratory variables in unanaesthetized (A) and anaesthetized (B) wild-type
and mutant mice during 100% O_2 and 3% and 5% CO_2 balanced O_2

		A. Unanaesthetized				B. Anaesthetized		
	n	100% O_2	3% CO_2	5% CO_2	n	100% O_2	3% CO_2	5% CO_2
		Respiratory rate (breaths min^{-1})				Respiratory rate (phrenic bursts min^{-1})		
Wild-type	12	211 ± 10.1	$244 \pm 11.6^*$	$278 \pm 12.9^*$	7	218 ± 17.5	$233 \pm 15.0^*$	$231 \pm 16.3^*$
Mutant	9	195 ± 11.5	216 ± 11.7	$272 \pm 13.7^*$	7	162 ± 10.9	$171 \pm 10.6^*$	$181 \pm 9.0^*$
		V_T (μl (g BW)$^{-1}$)				Amplitude of phrenic activity (a.u.)		
Wild-type	12	1.81 ± 0.13	$2.36 \pm 0.17^*$	$2.67 \pm 0.17^*$	7	1.3 ± 0.1	1.5 ± 0.2	1.9 ± 0.5
Mutant	9	2.04 ± 0.11	$2.62 \pm 0.09^*$	$3.05 \pm 0.18^*$	7	1.7 ± 0.2	1.7 ± 0.2	1.9 ± 0.3
		$\dot{V}_E$ (ml min^{-1} (g BW)$^{-1}$)				Minute neural respiration (a.u. min^{-1})		
Wild-type	12	0.40 ± 0.04	$0.57 \pm 0.05^*$	$0.74 \pm 0.04^*$	7	272.2 ± 33.1	351.7 ± 57.3	450.9 ± 131.9
Mutant	9	0.39 ± 0.02	$0.56 \pm 0.04^*$	$0.84 \pm 0.09^*$	7	282.4 ± 43.8	296.9 ± 35.2	353.1 ± 58.4

Data are means $\pm$ s.e.m. * P values < 0.05 within each group between control (100% O_2) and 3 or 5% CO_2
(paired t test).

These results suggest that NO generation is markedly impaired in the brainstem in mutant mice during hypoxia.

Respiratory responses to hypercapnia

To assess whether the alterations in respiratory responses are confined to low oxygen or if they extend to hypercapnic stimulus, respiratory responses to 3 and 5% CO_2 balanced O_2 were recorded in both awake and anaesthetized mice.

Minute ventilation increased significantly in response to 3% and 5% CO_2 in awake mutant and WT mice. These increases in respiration were due to increases in respiratory rate as well as tidal volume (Table 4A). On the other hand, anaesthetized animals (both WT and mutant mice) responded with increases in respiratory rate only during hypercapnia. As a consequence, overall changes in minute ventilation were not significant in both groups of
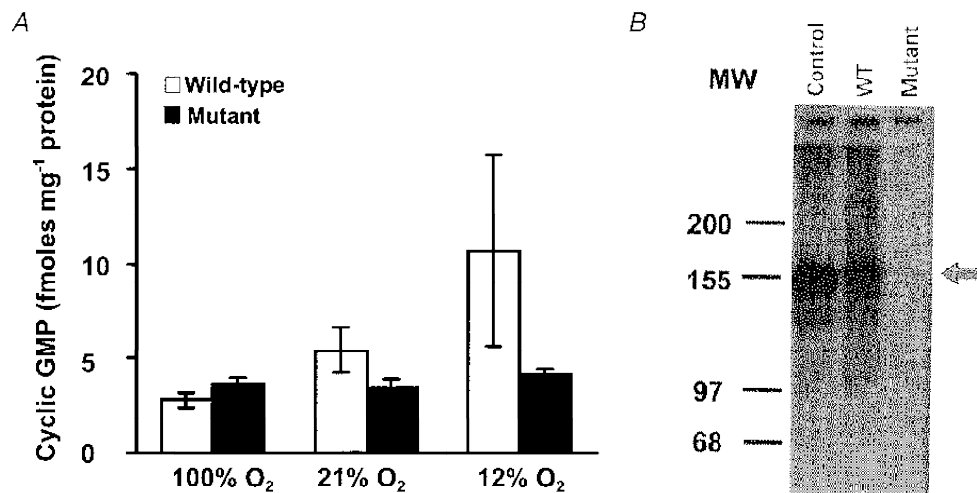

Figure 7. cGMP and Western blot analysis in wild-type and mutant mice

A, analysis of cGMP levels in brainstem during three levels of inspired oxygen, i.e. 100, 21 and 12% O_2, in wild-type and mutant mice. Data presented as means $\pm$ s.e.m. from 9 animals in each group. Note the increases in cGMP levels during 21 and 12% O_2 in wild-type (WT) mice ($P < 0.05$, paired t test), whereas they remained fairly constant in mutant mice ($P > 0.05$, paired t test). *B*, representative autoradiogram illustrating the absence of NOS-1 protein in tissue extracts of the brainstem from mutant mice. NOS-1 protein was analysed in the brainstem extracts by Western blot analysis using an antibody directed against NOS-1. The antibody recognizes the 155 kDa NOS-1 protein. Rat pituitary lysate was used as a positive control (Control). Note the presence of the NOS-1 protein in WT mice, but not in mutant mice.

anaesthetized mice (Table 4B). As shown in Fig. 8, the magnitude of respiratory responses to hypercapnia was the same in both awake and anaesthetized groups of mice ($P > 0.05$, ANOVA).

DISCUSSION

In the present study, we examined the role of endogenous NO generated by the neuronal isoform of NOS (NOS-1) in the control of respiration during hypoxia and hypercapnia. For this purpose we used mutant mice deficient in NOS-1. The advantage of using mutant mice over pharmacological inhibition of NOS activity is that it allows the study of NO produced by NOS-1 only, independently of other NOS isoforms. Our results provide evidence that NO derived from NOS-1 modulates respiration during hypoxia, but not during hypercapnia. Further, the results provide evidence for enhanced peripheral chemoreceptor sensitivity in mutant mice.

Respiratory responses to hypoxia are augmented in mutant mice

The advantages of studying awake as well as anaesthetized mice are that the former alleviates the effects anaesthesia may have on breathing, as well as allowing us to monitor changes in body metabolism and temperature, which are known to occur during hypoxia (Frappell *et al.* 1992;

Gautier, 1996). On the other hand, because of technical difficulties, changes in blood pressure and blood gases could not be monitored in this preparation and this was accomplished in the anaesthetized animal.

It is clear from the results from both types of preparations that mutant mice exhibited significantly greater respiratory responses during 21 and 12 % O_2 than WT control mice. Neither the changes in body temperature nor arterial blood pressure nor blood gases seem to account for the augmented respiratory response to hypoxia, because changes in these variables were comparable in both groups of mice. Furthermore, the P_{O_2} and P_{CO_2} obtained in the present study were comparable with those reported by other investigators in anaesthetized mice (Lahiri, 1975; Siemann *et al.* 1980). Significant differences in body metabolism, especially $\dot{V}_{O_2}$ during 21 and 12 % O_2, were noticed between WT and mutant mice. $\dot{V}_{O_2}$ decreased in both groups of mice on changing the inspired oxygen from 100 % to 21 and 12 % O_2. However, the magnitude of decrease was significantly greater in mutant mice. Since the increases in $\dot{V}_E$ were also greater in mutant mice during 21 and 12 % O_2, the ratio of $\dot{V}_E : \dot{V}_{O_2}$ is expected to be substantially higher in mutant mice under these conditions. This suggests that the increases in the ventilatory response to 21 and 12 % O_2 in mutant mice is much more substantial than the observed increases in $\dot{V}_E$ alone.
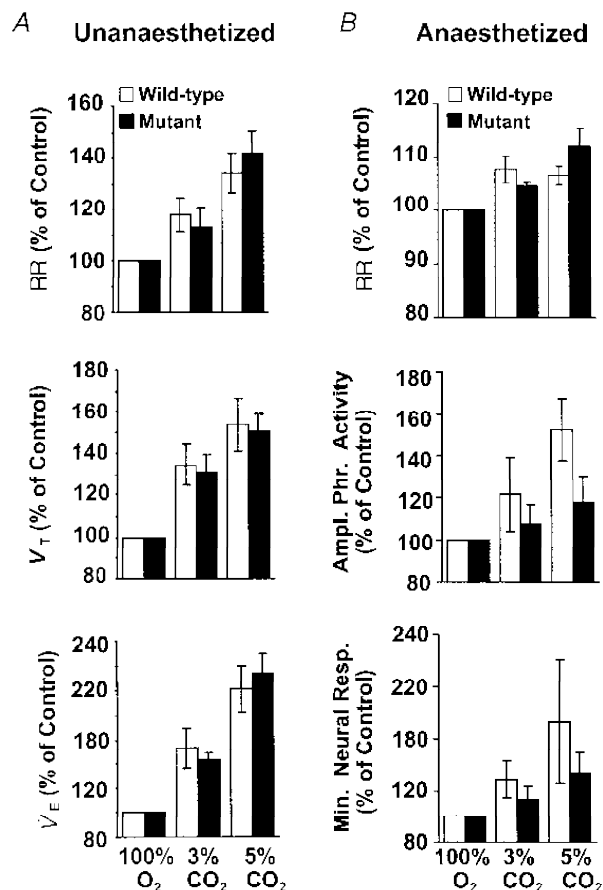

Figure 8. Comparison of hypercapnic (3 and 5% CO_2 balanced oxygen) respiratory responses in unanaesthetized (A) and anaesthetized (B) wild-type and mutant mice

The results are presented as percentage of 100 % O_2. A, in unanaesthetized mice, data are presented as means $\pm$ S.E.M. of 12 wild-type and 9 mutant mice. B, in anaesthetized mice, data are presented as means $\pm$ S.E.M. of 7 of each group. Note the magnitude of increase in respiratory variables in unanaesthetized and anaesthetized mice was similar in both groups of mice ($P > 0.05$, ANOVA).

Evidence for enhanced peripheral chemoreceptor sensitivity in mutant mice

Peripheral chemoreceptors, especially the carotid bodies, are necessary for stimulation of breathing during hypoxia. It is now well established that NOS-1 is present in the carotid body (Prabhakar *et al.* 1993; Wang *et al.* 1993; Grimes *et al.* 1995) and NO is inhibitory to the chemosensory activity (Prabhakar *et al.* 1993; Wang *et al.* 1994; Chugh *et al.* 1994; Trzebski *et al.* 1995). Since mutant mice are deficient in NOS-1, it is conceivable that NO production in the carotid body is either markedly reduced or absent. As a consequence, it is to be expected that the carotid body sensitivity will be enhanced in mutant mice. The following lines of evidence suggest that carotid body sensitivity is indeed increased in mutant mice. Firstly, the magnitude of respiratory depression caused by brief hyperoxia (i.e. the Dejours test) is more pronounced in mutant mice. Secondly, mutant mice have significantly greater respiratory responses to cyanide, a potent carotid body stimulant.

Gozal *et al.* (1996*a*) reported that respiratory responses to cyanide were unaffected following systemic administration of *S*-methyl-L-thiocitrulline (SMTC), a putative NOS-1 blocker, whereas N^{ω}-nitro-L-arginine methyl ester (L-NAME), which inhibits all three isoforms of NOS, potentiated the cyanide response. Based on these results, Gozal *et al.* (1996*a*) suggested that NO derived from NOS-1 may not function as an inhibitory messenger in the carotid body. However, our results showed a clear potentiation of cyanide responses in mutant mice. It may be that the relative contribution of NOS-1-derived NO may vary from rats to mice. Alternatively, acute effects of NOS-1 blockade may differ from chronic deficiency of NOS-1.

Evidence for impaired NO production in the brainstem during hypoxia in mutant mice

Previously, Ogawa *et al.* (1995) reported that L-citrulline levels in the brainstem increase during hypoxia in awake rats, and NOS inhibitors attenuate the ventilatory stimulation during hypoxia. Based on these results, these authors concluded that NO acting at the central neurons stimulates breathing in awake rats. Our findings that cGMP levels (index of NO production) are increased during hypoxia in WT mice are consistent with the notion that hypoxia increases NO generation in the brainstem neurons. In contrast to the results in WT mice, cGMP levels during hypoxia were unaffected in mutant mice. The fact that NOS-1 protein is absent in mutant mice suggests that NO generation during hypoxia is impaired. These results suggest that NO from NOS-1 may function as an inhibitory chemical messenger in the brainstem neurons. Such a notion seem supported by studies from Vitagliano *et al.* (1996) who reported that microinjections of NO donors (sodium nitroprusside (SNP) and 3-morpholinosydnonimine (SIN-1)) into the NTS neurons inhibits breathing in a dose-dependent manner. Thus our results with mutant mice do not support

the idea that NO is an excitatory chemical messenger in the brainstem neurons during hypoxia as suggested by Ogawa *et al.* (1995). The lack of inhibitory effects of NO peripherally, at the carotid body chemoreceptors, and centrally, in the brainstem neurons, contributed to the augmented respiratory response to hypoxia seen in mutant mice.

Respiratory oscillations in mutant mice

It is interesting to note that mutant mice deficient in NOS-1 displayed periodic oscillations in respiratory activity and this pattern of breathing was enhanced during hypoxia (Fig. 4). Cherniack *et al.* (1978) proposed that breathing instability may appear whenever controller gain is increased sufficiently due to increased chemoreceptor drive, and/or by damping the controller system. Our results suggest that carotid body sensitivity is enhanced in mutant mice. Thus, the respiratory oscillations in mutant mice may in part be due to increased peripheral chemoreceptor sensitivity, i.e. controller gain. Such a notion is further supported by the finding that respiratory oscillations were absent during 100% O_2, which is known to reduce peripheral chemoreceptor activity. In addition, Ogawa *et al.* (1995) suggested that NO might contribute to the stabilization of ventilation via its action on the brainstem neurons associated with the integration of afferent information from the peripheral chemoreceptors (controller). Thus, increased chemoreceptor sensitivity (increased controller gain), as well as altered activity of NTS neurons (controller), may have contributed to the respiratory oscillations in mutant mice.

NO derived from NOS-1 in CO_2 chemoreception

Unlike hypoxia, hypercapnic respiratory responses were found to be comparable in mutant and WT mice in awake and anaesthetized conditions. In the latter preparation, hypercapnic responses were relatively modest perhaps due to depressant effects of anaesthesia. Our findings are consistent with those by Gozal *et al.* (1996*b*) who reported that respiratory responses to CO_2 were unaffected by NOS inhibitors. It is well established that respiratory responses to CO_2 are primarily mediated by central chemoreceptors located near the ventral surface of medulla oblongata (Loeschcke, 1982). Whether NO derived from NOS-1 plays a role in CO_2 chemoreception at the central chemoreceptors, however, needs further investigation. Nonetheless, it is clear from the present results that the respiratory response to hypoxia, but not to hypercapnia, is selectively affected in mice deficient in NOS-1.

Physiological significance of NOS-1-generated NO

How might NOS-1 contribute to the control of breathing during hypoxia? It has long been postulated that a haem-containing protein(s) are involved in sensing arterial blood oxygen. Relevant to this idea is the fact that the NOS-1 enzyme is a haem-containing protein and molecular oxygen is essential for its activity (Leone *et al.* 1991). We have previously reported that NOS activity in the carotid bodies

is markedly reduced during hypoxia (Prabhakar *et al.* 1993) and Rengasamy & Johns (1991) obtained similar results in brain extracts. Furthermore, NOS-1 has been shown to have the highest K_m value for oxygen compared with the other NOS isoforms (Rengasamy & Johns, 1996), suggesting that NOS-1 is most sensitive to oxygen. In addition, Abu-Soud *et al.* (1996) recently made a detailed biochemical analysis of NOS-1 with regard to its sensitivity to oxygen. These investigators provided evidence that NO synthesis from NOS-1 is coupled to physiological oxygen concentrations. The fact that the respiratory responses to hypoxia are selectively altered in NOS-1-deficient mice is consistent with the idea that NO derived from NOS-1 plays an integral role in the respiratory response to hypoxia, acting peripherally at the sensor (carotid body) as well as centrally at the brainstem neurons.

In summary, the results of the present study demonstrate that respiratory responses to low oxygen are selectively augmented in mutant mice deficient in NOS-1, suggesting that NO generated by NOS-1 is an important physiological modulator of respiration during hypoxia. Our observations further suggest that NOS-1-deficient mice offer an excellent animal model to delineate the role of NO generated from NOS-1 in the regulation of respiration.

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