Effects of chronic hypoxia on MK-801-induced changes in the acute hypoxic ventilatory response

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Effects of chronic hypoxia on MK-801-induced changes in the acute hypoxic ventilatory response. J Appl Physiol 99: 2108–2114, 2005. First published August 18, 2005; doi:10.1152/japplphysiol.01205.2004.—Chronic hypoxia increases the sensitivity of the central nervous system to afferent input from carotid body chemoreceptors. We hypothesized that this process involves N-methyl-D-aspartate (NMDA) receptor-mediated mechanisms and predicted that chronic hypoxia would change the effect of the NMDA receptor blocker dizocilpine (MK-801) on the poikilocapnic hypoxic ventilatory response (HVR). Male Sprague-Dawley rats were studied before and after acclimatization to hypoxia (70 Torr inspiratory PO2 for 9 days). We measured ventilation (Vi) and the HVR before and after systemic MK-801 treatment (3 mg/kg ip). MK-801 resulted in a constant respiratory frequency (~175 min−1) during acute exposure to 10% and 30% O2 before and after acclimatization. MK-801 had no effect on tidal volume (Vt) before acclimatization, but it significantly decreased Vt when the animals were breathing 10% O2 after acclimatization. The net effect of MK-801 was to eliminate the O2 sensitivity of Vi before (via changes in respiratory frequency) and after (via changes in Vt) acclimatization. Hence, chronic hypoxia altered the effect of MK-801 on the acute HVR, primarily because of increased effects on Vt. This indicates that changes in NMDA receptor-mediated neurotransmission may be involved in ventilatory acclimatization to hypoxia. However, further experiments are necessary to determine the precise location of such plasticity in the central nervous system.

chronic hypoxia; control of breathing; hypobaric hypoxia; hypoxic ventilatory response; rat

A growing body of literature suggests an important role for N-methyl-D-aspartate (NMDA) and non-NMDA glutamatergic receptors in the regulation of the HVR (4, 22, 33). Ohtake et al. (37) demonstrated that intraperitoneal injections of the NMDA receptor channel blocker MK-801 (dizocilpine) reduced the HVR in awake rats. Mizusawa et al. (34) reported that microinjections of MK-801, through a chronically implanted cannula, into the caudal NTS attenuated the ventilatory responses during hypoxia (34). Furthermore, simultaneous antagonism of NMDA and non-NMDA receptors, within the NTS, abolished the ventilatory responses to carotid body stimulus in anesthetized rats (43).

On the basis of the effects of NMDA receptor blockade on the acute HVR, we hypothesized that changes in NMDA receptor-mediated glutamatergic neurotransmission contribute to the increase in the CNS gain of the HVR during chronic hypoxia (13). We predicted that the effects of global NMDA receptor blockade on the acute HVR in chronically hypoxic rats would be significantly different from those in control rats maintained under normoxic conditions. We tested this hypothesis by measuring the effects of chronic hypoxia on the acute poikilocapnic HVR before and after systemic treatment with MK-801.
MATERIALS AND METHODS

Experimental animals. Male Sprague-Dawley rats (250–350 g; Charles River) were housed in standard rat cages in a vivarium and fed ad libitum a standard rat diet. A 12:12-h light-dark cycle was maintained within the vivarium. All experiments were approved by the University of California, San Diego, Animal Care and Use Committee. The experiments conformed to national standards for the care and use of experimental animals as well as the American Physiological Society’s “Guiding Principles in the Care and Use of Animals.” Animals were divided into two experimental groups: one was exposed to chronically hypoxic conditions (n = 10; see below), and the other was maintained under control (normoxic) conditions (n = 10).

Animal preparation. Breathing was measured using the barometric method of plethysmography (see below), which requires a measurement of body temperature for accurate calculation of VT. Body temperature was measured with abdominally implanted radio telemetry devices (Minimitter) that emit a radio frequency proportional to body temperature. The telemetry was calibrated, before implantation, at 34–42°C.

For implantation of these telemetry devices, animals were initially anesthetized with 3.5% isoflurane in O2 and maintained under anesthesia with 2–2.5% isoflurane in O3. Rodent hair clippers were used to remove fur from an ~2.5 × 4.0 cm area of the abdomen, and the underlying skin was cleansed with surgical scrub and alcohol before application of a 10% solution of Betadine (povidone-iodine). A 2-cm-long incision was made in the rostral-to-caudal axis, and a sterile telemeter was inserted into the body cavity. The telemeter was precoated with paraffin wax to protect it from body fluids. The muscle and skin were sutured separately, and the animal recovered from anesthesia. Experiments commenced 5 days after surgery.

Chronic hypoxia. One group of rats was exposed to chronic hypoxia for 9 days in a hypobaric chamber maintained at 0.5 atm (380 mmHg), which approximates exposure to 10% O2 at sea level and mimics the conditions of chronic hypoxia encountered at ~6,000 m above sea level. Animals, within individual cages, were placed into the hypobaric chamber, and the pressure was lowered from 1.0 to 0.5 atm over a 15-min period. The chamber was opened once daily (~10 min) for regular cage maintenance or when it was necessary to remove animals for experimentation. The hypobaric chamber was maintained in the same vivarium that housed the control animals.

Plethysmography. Breathing was measured using the barometric method of plethysmography modified for continuous flow (1, 27, 31, 36). Animals were placed in a sealed plethysmograph (7 liters) supplied with an inflowing gas mixture (2 l/min) from an electronic gas-mixing pump (model GF4, Cameron Instruments). O2, CO2, and N2 were blended to create the desired gas mixtures. Gas was removed from the chamber, at the same rate as it entered, through a needle valve attached to a vacuum pump. A water manometer was used to monitor pressure in the plethysmograph. We did not use a reference chamber but, rather, adjusted the vacuum valve to maintain chamber pressure near atmospheric level (~0.5 cmH2O monitored by the water manometer) (1). The inflowing gas entered the chamber through a tube (7 cm long, 1 cm ID) that was filled with similar lengths of PE-50 tubing. Hence, gas entered the plethysmograph through a high impedance to reduce the loss of pressure signals. Air temperature was measured with a thermostir and relative humidity was monitored with a digital hygrometer, both of which were sealed within the plethysmograph.

On inspiration, the air that is taken into the lungs is warmed and humidified, causing it to increase in volume, which, in turn, causes a rise in pressure. Therefore, breathing is measured from the change in pressure associated with the change in volume resulting from the warming and humidification of air during inspiration (12). These pressure changes were monitored with a differential pressure transducer (model DP45, Validyne) referenced to atmosphere. Output from the transducer demodulator was recorded on a digital data acquisition system (model DT-2801, National Instruments; 200-Hz sample rate) and a Gould chart recorder. Calibration pulses (0.5 and 1 ml) were generated by a gas-tight syringe and injection of air pulses into the plethysmograph at a rate similar to the rat’s inspiratory duration (Ti).

Experimental protocol: acute breathing trials. After 9 days of chronic hypoxia or control normoxia, animals were weighed and placed into the plethysmograph for a 1-h acclimation period. During this period, the plethysmograph was ventilated with 21% O2-0% CO2 for the normoxic control and 10% O2-0% CO2 for the chronically hypoxic animals. After the 1-h acclimation period, the inflowing gas composition was changed every 15 min to alternate between 30% and 10% O2, which were chosen as standard stimuli to obtain a robust two-point HVR.

Previous experiments from this laboratory have shown that 30% O2 reduces arterial chemoreceptor drive to negligible levels (1, 13). After the inspired gas was changed, washout of the plethysmograph required ~1 min to attain a new, stable level. O2 and CO2 levels within the plethysmograph were monitored using O2 and CO2 analyzers (models OM-11 and LB-2, respectively, Beckman).

After the animals were exposed to the appropriate gas mixtures required to produce a two-point HVR, they were removed from the plethysmograph and given an intraperitoneal injection of MK-801 [(dizocilpine, (5R,10S)-(+) -5-methyl-10,11-dihydro-5H-dibenzo(a,d)-cyclohepten-5,10-imine, maleate salt; Sigma; 3 mg/kg in 1.0 ml of sterile 0.9% NaCl). The dose was chosen on the basis of previous experiments in which the effects of systemically administered MK-801 on the acute HVR were examined (17, 35, 37). After an additional 1 h within the plethysmograph, the animals were exposed to the gases described above.

Data analysis. Breathing was measured for the last 2 min of a 15-min exposure to the desired gas composition. Each recording period was saved to a digitized file and onto chart paper. Each data file was analyzed using analysis software (design-in-house) to determine fR (breaths/min), VT (ml/kg), Ti, and the total time of the respiratory cycle (Tr). VT calculations were based on the equation described by Drorbaugh and Fenn (12). Minute ventilation (Vt, ml·kg−1·min−1) was calculated as the product of fR and VT. Expiratory time (Te, s) was calculated by subtracting Ti from Tr. An index of inspiratory drive was determined by calculating the ratio of Vt to Ti.

Values for fR, VT, and Ti during 30% and 10% O2 breathing are shown in Fig. 1. Difference in fR, VT, and Ti between 10% O2 and 30% O2 breathing (i.e., the magnitude of the HVR) before and after administration of MK-801 are shown in Fig. 2. The effect of MK-801 on the HVR, as the pre-MK-801 HVR minus the post-MK-801 HVR [(pre-MK-801 O2 − 30% O2) − (post-MK-801 10% O2 − 30% O2)], is shown in Fig. 2, insets.

Statistical analysis. All statistical testing, including the determination of normality and equal variance, was performed using commercial software (SigmaStat 3.0, SPS). If the data were normally distributed, parametric tests were applied; if the data were not normally distributed, nonparametric tests were applied. After all ANOVA tests, the appropriate post hoc multiple comparison test was selected, based on the data, by the software.

Within the normoxic control and chronically hypoxic groups, a two-way repeated-measures ANOVA, followed by a post hoc multiple comparison test, was used to determine whether there was a statistically significant difference between the values at 30% O2 and 10% O2 and between the values before and after MK-801 treatment. A two-way non-repeated-measures ANOVA (with normoxic control as the 2 factors) followed by a multiple comparison test was used to determine whether there were significant differences between the normoxic control and chronically hypoxic groups. The magnitude of the acute hypoxic response (i.e., the value at 10% O2 minus the value at 30% O2) before and after MK-801 in the control and chronically hypoxic groups was compared with the magnitude of the acute hypoxic response in the chronic hypoxic group.
(pre-MK-801, 10% O₂ / H₁₀₀₂ - post-MK-801, 30% O₂ / H₁₀₀₂) - (post-MK-801, 10% O₂ / H₁₀₀₂ - post-MK-801, 30% O₂ / H₁₀₀₂)), in the control and chronically hypoxic groups was compared with a t-test. In all cases, the fiducial limit of significance was taken to be 5% (P < 0.05).

RESULTS

Effects of chronic hypoxia before MK-801 treatment. When rats were breathing 30% O₂ before MK-801 treatment, chronic hypoxia caused an increase in fR, Vr, and Vi (Fig. 1). The fR increased as a result of a significant decrease in TE (0.41 ± 0.03 and 0.24 ± 0.01 s for control and chronic hypoxia, respectively) with no significant change in TI (0.24 ± 0.02 s and 0.20 ± 0.03 s for control and chronic hypoxia, respectively). Chronic hypoxia caused a significant increase in Vr/Ti (21.0 ± 1.7 and 41.8 ± 5.7 ml·kg⁻¹·s⁻¹ for control and chronic hypoxia, respectively) while breathing 30% O₂.

While rats were breathing 10% O₂ before MK-801 treatment, chronic hypoxia caused a significant increase in Vr and Vi (Fig. 1) but no significant changes in fR (Fig. 1; P = 0.066), Ti (0.16 ± 0.01 and 0.15 ± 0.03 s for control and chronic hypoxia, respectively), and Te (0.23 ± 0.02 and 0.17 ± 0.02 s for control and chronic hypoxia, respectively, P = 0.072). Chronic hypoxia significantly increased Vr/Ti (33.0 ± 1.4 and 60.1 ± 6.5 ml·kg⁻¹·s⁻¹ for control and chronic hypoxia, respectively) while breathing 10% O₂ before MK-801. Before MK-801, chronic hypoxia did not significantly increase the magnitude of the poikilocapnic HVR (Fig. 2).

Effects of MK-801. MK-801 abolished the sensitivity of fR to 10% O₂ in rats before and after acclimatization to chronic hypoxia (● and ■) and MK-801 treatment (■ and ▼) compared with normoxic controls (○ and △). Values are means ± SE.

*Significantly different from 30% O₂; #significantly different from pre-MK-801; †significantly different from control (P < 0.05).
MK-801 had no effect on VT in control rats (Fig. 1C) but significantly decreased VT during exposure to 10% O2 in the chronically hypoxic rats (Fig. 1D). In other words, the effects of MK-801 on VT during acute hypoxia were altered by chronic hypoxia.

The net effect of MK-801 on ventilation was to eliminate the O2 sensitivity of V˙I before and after chronic hypoxia. However, similar to the effects on fR, this was caused by a significant increase in V˙I while breathing 30% O2 before acclimatization (Fig. 1E) vs. a significant decrease in V˙I while breathing 10% O2 after acclimatization (Fig. 1F).

MK-801 significantly decreased the magnitude of the overall HVR in control and chronically hypoxic rats (Fig. 2C). This was caused by decreases in the fR component of the HVR with MK-801 in control and chronically hypoxic rats (Fig. 2A) and a decrease in the VT component of the HVR in the chronically hypoxic rats (Fig. 2B).

Although the effect of MK-801 on the fR component of the HVR was not changed by chronic hypoxia (Fig. 1, A and B, and Fig. 2A), acclimatization caused a significant attenuation of the MK-801-induced decreases in the Ti (P = 0.009) and Te (P = 0.002) components of the HVR (data not shown). The V˙I/Ti component of the HVR was significantly augmented (P = 0.028) by MK-801 in chronically hypoxic vs. control rats.

The insets in Fig. 2 show the magnitude of change in the various components of the HVR with MK-801 treatment calculated using the following equation: pre-MK-801 HVR – post-MK-801 HVR. The magnitude of the change in the HVR with MK-801 was significantly greater in chronically hypoxic than in control rats (Fig. 2C, inset: 515 ± 51 vs. 341 ± 57 ml·min⁻¹·kg⁻¹) because of the effect of MK-801 on VT after chronic hypoxia (Fig. 2B, inset).

**DISCUSSION**

**Critique of the methods.** We hypothesized that chronic hypobaric hypoxia would alter the effects of systemic NMDA receptor blockade on the acute HVR. This is based on our hypothesis that changes in NMDA-mediated glutamatergic neurotransmission contribute to the increase in the sensitivity of the CNS to afferent input from carotid body chemoreceptors during VAH (13). Systemic (intraperitoneal) application of an NMDA receptor channel blocker (MK-801) represents an initial step to address this question, with the obvious limitation that systemic MK-801 treatment cannot target any specific region of the brain. However, the effect of systemic MK-801 on the acute HVR was changed by chronic hypoxia, which is consistent with NMDA receptor plasticity contributing to VAH.

Using radioactively labeled MK-801, Coles et al. (8) identified a number of sites in the pons and medulla that contain NMDA-type glutamate receptors. These sites include the ventrolateral and dorsolateral pons as well as the parapyramidal nuclei, the NTS, and the reticular formation. It is possible that systemic MK-801 treatment could offset effects in different regions of the brain that, when taken together in the intact animal, result in no changes in the ventilatory responses. For example, Harris and Milsom (24) observed, in anesthetized and vagotomized ground squirrels, that systemic MK-801 treatment did not change fR. However, their MK-801 injections directly into the pontine respiratory group decreased fR.
Our results are consistent also with the possibility that blocking NMDA receptors throughout the CNS of an awake rat unmasks a fundamental ventilatory drive that bypasses or dominates the ventilatory reflex responses to acute hypoxia as well as plasticity from chronic hypoxia. Our experimental design predicted that if NMDA receptor-mediated processes in the acute HVR were upregulated by chronic hypoxia, then MK-801 treatment would have a proportionally greater effect on the HVR after chronic hypoxia. Conversely, if chronic hypoxia decreased the role of NMDA receptor-mediated regulation of the O2 chemoreflex, then the HVR would be less sensitive to MK-801 in chronically hypoxic than in control rats. Such NMDA receptor plasticity may have occurred during acclimatization, but it might not be detected if another control system dominates ventilatory drive after MK-801. The observation that Vt after MK-801 was the same in control and acclimatized rats was surprising. The fact that the HVR changed in different ways before vs. after chronic hypoxia to result in this stereotypical level of Vt with MK-801 suggests that NMDA receptors normally prevent the expression of a fundamental ventilatory drive that is not sensitive to O2.

Comparison with the literature. In our normoxic control rats, systemic administration of MK-801 caused a significant increase in fR (from 98 to 173 min⁻¹) and, hence, Vt, while the rats were breathing 30% O2. However, when the gas was changed to 10% O2, fR and Vt did not increase after MK-801. It is possible that the initial rise in fR after MK-801 treatment resulted from inhibition or alteration of NMDA-mediated processes within the pontine respiratory group (nucleus parabrachialis medialis and Kölliker-Fuse nucleus) that regulate breath timing (17, 30). However, systemically administered MK-801 normally results in the prolongation of inspiration (i.e., apneas) in vagotomized animals or those in which lung inflation has been withheld experimentally (7, 16, 18). If the vagus nerves are intact, as was the case in the present study, systemic MK-801 treatment does not appear to alter inspiratory timing (7, 16, 18). In the present study, the MK-801-induced decrease in Tl during exposure to 30% O2 suggests that systemic MK-801 altered fR in our rats by a mechanism different from NMDA-mediated inspiratory termination within the pons.

A previous study (37) that examined the effects of systemic MK-801 treatment on the HVR in unacclimatized control rats found effects in acute hypoxia but not normoxia. In that study, fR with 21% O2 increased from 95 to 120 min⁻¹ after intravenous administration of MK-801 (Fig. 2 from Ref. 37) compared with an increase from 98 to 173 min⁻¹ with 30% O2 in our study. By contrast, MK-801 with 10% O2 decreased fR from 165 to 45 min⁻¹ in the other study but did not change fR in our study (Fig. 1A). In the previous study (37), the acute hypoxic trial was performed 30 min after MK-801 treatment; in the present study, the acute hypoxic trials began 60 min after MK-801. Foutz et al. (16) reported that the effects of intravenous MK-801 were long-lasting and that variables associated with respiration did not return to pre-MK-801 levels within the 4- to 6-h time course of their studies, so we do not suspect that different time points explain the differences between our studies.

It is possible that the differences between the results from this study and those reported by Ohtake et al. (37) were due to different strains of rats (Sprague-Dawley in the present study and Wistar in the previous study). Other authors (25, 42) reported differences in respiratory chemoreflexes between these two strains of rats. Indeed, Kobayashi et al. (28) reported that identical levels of electrical stimulation of the carotid sinus nerve caused different effects on fR and VT in Sprague-Dawley vs. Wistar rats. In the Sprague-Dawley rats, carotid sinus nerve stimulation caused a 202 ± 14% increase in fR but little change in VT (104 ± 15% of control). By contrast, carotid sinus nerve stimulation in Wistar rats caused a 139 ± 20% increase in fR and a 140 ± 20% increase in VT. The differences suggest that the peripheral O2 chemoreflex consists of a greater fR in the Sprague-Dawley than in the Wistar rats. As such, it is perhaps not surprising that MK-801 has a greater, or different, effect on fR in Sprague-Dawley vs. Wistar rats.

Connelly et al. (9) measured ventilation after intravenous injections of MK-801 in anesthetized Wistar and Sprague-Dawley rats. They concluded that the breathing pattern in Sprague-Dawley rats is more sensitive to interference with NMDA-mediated mechanisms. Borday et al. (5) reported that the same dose of MK-801 used in this study increased breathing (fR and VT) in adult mice but had the opposite effect in neonatal mice and adult cats. Cassus-Soulanis et al. (7) suggested that the increase in breathing associated with systemic MK-801 treatment results from a stimulatory effect of MK-801 on forebrain structures controlling breathing (25). Harris and Milsom (23) reported that systemic (intravenous) MK-801 treatment also increased (138%) fR, but not VT, in anesthetized ground squirrels. They also found that acute hypoxia (10% O2) after MK-801 did not result in any further increase in breathing (fR, VT, and Vt). These observations (23) are similar to our results.

Differential effects of MK-801 in control and chronically hypoxic rats. The effects of MK-801 on ventilation and the HVR after acclimatization to hypoxia have not been studied previously. After chronic hypoxia, we observed a change in the effect of MK-801 on VT. This could reflect a change in NMDA receptor-mediated mechanisms that are important for the acute HVR and plasticity in ventilatory chemoreflexes with chronic hypoxia (see Critique of the methods). Alternatively, it could reflect the unmasking of a dominant ventilatory drive after MK-801 with or without changes in NMDA receptor-mediated mechanisms in the arterial chemoreflex resulting from chronic hypoxia. Quantification of the effects of MK-801 on the HVR showed a significant increase in the effect of NMDA receptor blockade with chronic hypoxia (Fig. 2C). This was primarily an effect of MK-801 on VT (Fig. 2B; P = 0.063).

Potential mechanisms of plasticity. Dwinell and Powell (13) demonstrated that chronic hypoxia caused an increase in phrenic nerve output in response to carotid sinus nerve stimulation. In other words, independent of carotid body O2 sensitivity, an equivalent level of carotid sinus nerve stimulation resulted in greater phrenic nerve activity in chronically hypoxic than in normoxic rats. We hypothesized that this may involve changes in NMDA receptor-mediated mechanisms in the arterial chemoreflex pathway. Our results are consistent with this hypothesis, although they cannot prove it because of the insensitivity of ventilation to O2 after MK-801 and the global nature of NMDA receptor blockade with systemic MK-801. However, the literature does support the idea of NMDA receptor plasticity with chronic hypoxia.
Reeves et al. (40) recently examined the effects of sustained chronic hypoxia (30 days; normobaric; 10% O2) on NMDA receptor expression in the dorsocaudal brain stem of adult male Sprague-Dawley rats from Charles River. Reeves et al. demonstrated that increased expression of the NR1 subunit of the NMDA-type glutamate receptor occurs in the early stages of the hypoxic period (from ~1 h to 3 days). However, NR1 expression returned to baseline levels by days 3–7 of sustained hypoxia, which is earlier than the time point we studied for chronic hypoxia (i.e., 9 days). Sustained hypoxia did not cause a change in expression for the NR2A or NR2B subunit of the NMDA receptor. Kobayashi and Millhorn (29) demonstrated that 7 days of exposure to 5% O2 caused an attenuation of NMDA-induced Ca2+ accumulation in PC12 cells via down-regulation of the NMDA receptor subunit 1.

Chronic hypoxia may also cause changes in other important systems involved in the HVR. Gozal et al. (20) reported that platelet-activating factor within the dorsocaudal brain stem is involved in regulating Vt during normoxia and hypoxia, with no effects during hypercapnia. Schmitt et al. (41) demonstrated that VAH in rats was followed by an increase in tyrosine hydroxylase activity and norepinephrine turnover in the caudal NTS and that the increase in ventilation observed during VAH was significantly correlated to tyrosine hydroxylase activity in the NTS. It is possible that chronic hypoxia may also elicit changes in protein kinase C function. Several studies have demonstrated that this enzyme, within the NTS, plays an important role in the acute HVR (19, 21). Furthermore, inhibition of tyrosine kinase in the dorsocaudal brain stem attenuates the acute HVR in conscious rats (10). It is possible that changes in any of these factors, and others, may contribute alone or in concert with other factors, in the genesis of VAH.

In conclusion, the results show that systemic NMDA receptor blockade with MK-801 eliminates the HVR in normoxic control and chronically hypoxic rats. A surprising result was a constant fR after MK-801 with or without carotid body stimulation (breathing 10% O2 and 30% O2, respectively) before and after acclimatization to chronic hypoxia. Consequently, MK-801 eliminated ventilatory sensitivity to acute changes in O2 before and after acclimatization. However, because chronic hypoxia increased ventilation, MK-801 caused a significantly greater decrease in the HVR after acclimatization, primarily because of increased effects on Vt. This is consistent with NMDA receptor-mediated mechanisms in the CNS contributing to VAH. However, because of the fixed fR after systemic MK-801, additional experiments are necessary to prove that plasticity of NMDA receptors specifically in the arterial chemoreflex pathway contributes to VAH.

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