Role of GABA within the nucleus tractus solitarii in the hypoxic ventilatory decline of awake rats

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The ventilatory response to acute hypoxia (hypoxic ventilatory response; HVR) in humans and some other mammalian species is biphasic (26, 39). The initial rise in ventilation (early phase of the HVR) is followed by a marked decline after several minutes to values above the prehypoxic level. This decline in ventilation has been termed “ventilatory roll-off” or “hypoxic ventilatory decline” (HVD) (5, 24).

The underlying mechanisms of this decline have not been completely elucidated. However, various neurotransmitters or modulators released during hypoxia in the central nervous system (CNS), for example, adenosine (40), γ-aminobutyric acid (GABA) (15), opioids (38), and so on, have been suggested as possible mediators of the HVD. In particular GABA, which is a major inhibitory amino acid in the CNS, seems to play an important role in the HVD. Intracerebroventricular perfusion of GABA decreased the ventilation (17). Systemic administration of bicuculline, one of the GABA receptor antagonists, abolished the HVD (13).

Hypoxia is sensed by the peripheral chemoreceptors, and removal of the peripheral chemoreceptors abolishes the HVR: both the early phase of HVR and HVD (21). The chemoreceptor afferent input is conveyed to the nucleus tractus solitarii (NTS) via the carotid sinus nerve (12, 34). The NTS is the first relay nucleus of respiratory, cardiovascular, and other autonomic nervous regulations (1, 23, 27, 34). In particular, NTS has an important role in the early phase of the HVR (23, 27).

A histological study showed that GABA exists in the NTS (32), and some reports suggested that GABA in the NTS has a depressant effect on ventilation. Champagnat et al. (4) reported that application of GABA or a GABA agonist to the cat medullary respiratory neurons inhibited the neuronal discharge. Iontophoretic application of GABA into the NTS abolished or markedly reduced the neuronal activity evoked by electrical stimulation of the carotid sinus nerve (22). From these findings, GABA in the NTS may be related to the occurrence of the HVD in response to peripheral chemoreceptor stimulation. In this study, we tested our hypothesis that the GABAergic mechanism modulates the HVD within the NTS and that this mechanism requires peripheral chemoreceptor stimulation in unanesthetized, freely moving rats.

METHODS

This study was approved by the Animal Experiment Review Committee of Tohoku University School of Medicine. All experiments were performed using unanesthetized, freely moving Sprague-Dawley (S-D) rats. We used anesthesia only during the following surgical preparations.

Animal Preparations

The surgical preparations were performed on anesthetized (Nembutal, 40 mg/kg ip; Abbott, North Chicago, IL) male S-D
rats (283 ± 15 g, mean ± SD). The depth of anesthesia was assessed by the disappearance of the motion response to limb pinching. The initial dose of Nembutal was sufficient for completion of the operation.

**Guide cannula implantation.** For microdialysis or micro-injection studies, a guide cannula made of stainless steel [0.5-mm outer diameter (OD)] was implanted just above the NTS with the use of a stereotaxic apparatus (Narishige, Tokyo, Japan). The coordinate was set at 8.6 mm caudal, 0.5 mm lateral to bregma, and 10.0 mm below the upper skull surface at the point of bregma at an angle of 30° caudal from the vertical (30). The dialysis probe or the microinjection needle, being 1 mm longer than the guide cannula, was projected into the NTS. The cannula was attached to screws placed in the skull with dental acrylic resin (GC, Tokyo, Japan). In six rats, the guide cannula was implanted into the medullary reticular nucleus for microdialysis outside of the NTS. The animals were then kept in a cage with free access to food and water for at least 24 h until the start of the experiments.

**Peripheral chemodenervation.** Carotid body denervation (CBD) was performed in CBD rats 3–5 days before the experimental day. The operation was performed through a ventral cervical midline incision. The bilateral carotid sinus nerves were carefully isolated and cut just before the glossopharyngeal nerve. The CBD procedure was considered successful when no increase in ventilation was observed on hypoxic exposure after recovery from anesthesia. In sham-operated rats, a midline neck incision was made and the carotid bifurcation was isolated, but all nerve connections were left intact. A guide cannula was also implanted into these CBD and sham-operated rats by use of the same method described above.

**Experimental Preparations**

**Ventilation measurements.** Ventilation was measured by use of the barometric methods of plethysmography as described in previous reports (2, 23). The rat was placed in a 7-liter Plexiglass chamber that was connected to a reference chamber. The animal chamber had inlet and outlet tubes, allowing for constant background flow of humidified room air. Inlet and outlet flows were maintained to be equal and constant. Calibration of the volume was repeated during each experiment by injection of 0.2 ml of air into the chamber, using a gas-tight syringe (1 ml). The respiratory frequency was calculated directly from the ventilation-induced pressure swings. Tidal volume was obtained as a function of the pressure difference between the two chambers. The temperature in the chamber was continuously measured by a thermometer. The equation to calculate tidal volume is described in previous reports (2, 23). The rat was placed in a 7-liter Plexiglass chamber that was connected to a reference chamber. The animal chamber had inlet and outlet tubes, allowing for constant background flow of humidified room air. Inlet and outlet flows were maintained to be equal and constant. Calibration of the volume was repeated during each experiment by injection of 0.2 ml of air into the chamber, using a gas-tight syringe (1 ml). The respiratory frequency was calculated directly from the ventilation-induced pressure swings. Tidal volume was obtained as a function of the pressure difference between the two chambers. The temperature in the chamber was continuously measured by a thermometer. The equation to calculate tidal volume is described in previous reports (2, 23).

**Microdialysis study.** At least 24 h after the guide cannula implantation, a microdialysis probe (1-mm membrane tip, 220-μm OD, A-I-12-01; Eicom, Kyoto, Japan) for the measurement of the extracellular GABA concentration ([GABA]_o) was carefully inserted into the NTS through the guide cannula and fixed by a screw apparatus. The rat was then placed in the 7-liter acrylic chamber. A polyvinyl tube, connected to the microdialysis probe, was attached to a microsyringe outside the chamber. Through this tube-probe, Ringer solution (Na^+ 147 meq/l, K^+ 4.0 meq/l, Ca^{2+} 4.5 meq/l, Cl^- 155.5 meq/l) was perfused at a rate of 2 μl/min, and the dialysate was collected every 15 min.

**HPLC method for GABA detection.** The derivitizing agent, 10 ml of 4.0 mM O-phthalaldehyde (OPA) solution with 4 μl of 2-mercaptoethanol (2-ME) (Wako Pure Chemical, Osaka, Japan), was freshly prepared daily before the experiment. Each sample of dialysate (20 μl) was mixed with 4 mM OPA solution containing 2-ME (10 μl) by an autosampling injector (231 XL, Gilson Medical Electronics) at 10°C for 10 min. The dialysate was then injected into an HPLC system (EP-10, Eicom) with an electrochemical detector (ECD-100, Eicom). The mobile phase (including 5 mg/l EDTA-Na2) for GABA detection consisted of 60% 0.05 M PBS (pH 3.7) and 40% methanol. The GABA peak was identified and quantified compared with the peak of the known concentration of GABA solution. The peak data were collected and calculated by an A-D converter (Power Chrom, AD Instruments) and computer software (Power Chrom version 2.09, AD Instruments).

**Microinjection tests.** A fine stainless steel microinjection needle (350-μm OD) was carefully inserted into the guide cannula implanted in the NTS. The polyvinyl tube connected to the microinjection needle was attached to a Hamilton microsyringe outside the chamber. Drug or vehicle (PBS) was microinjected into the unilateral NTS with the use of the microinjection pump (CMA-100; CMA, Stockholm, Sweden).

**Drugs Used for Microinjection Studies**

**GABA receptor agonists.** As the GABA\(_A\) receptor agonist, muscimol (150 pmol; Wako Pure Chemical) was used, and, as the GABA\(_B\) receptor agonist, baclofen (400 pmol; Wako Pure Chemical) was used.

**GABA receptor antagonists.** We used (-)-bicuculline methiodide (bicuculline) as the GABA\(_A\) receptor antagonist. As GABA\(_B\) receptor antagonists, hydroxysaclofen (saclofen, 400 pmol; Toiris Cookson) and CGP-35348 (CGP, 2.5 nmol) were also used. CGP was kindly supplied by Novartis-Pharma (Basel, Switzerland).

All the drugs were dissolved into the PBS solution (pH = 7.4). A quantity of 100 nl of each drug solution (the final doses were as described above) was microinjected. For the control study, the PBS at the same volume was microinjected.

**Experimental Protocols**

The animals (n = 87) were divided into two major groups: the microdialysis study group (n = 21) and the microinjection study group (n = 66).

**Microdialysis Group**

For the microdialysis group, n = 21.

**Group 1: effect of hypoxia on [GABA]_o in the NTS.** For group 1, n = 15. To examine [GABA]_o in the NTS during hypoxic load, microdialysis was performed. The perfusion was continued for 3 or 4 h in room air to ensure that [GABA]_o of several samples had equilibrated, and then the experiments were started. We defined the baseline [GABA]_o as the mean value of [GABA]_o in the first three of four samples collected 1 h before hypoxia. After the measurement of [GABA]_o in the NTS in the room air condition just before the hypoxia, poikilocapnic hypoxia ([P_O2 = 10%] was induced
by N2 gas mixed with the background room airflow for 45 min. The flow of the gas mixture was controlled to keep the FO2 in the chamber at 10% and the FCO2 <0.6% for 45 min. The dialysate was collected every 15 min. During the 45 min of hypoxia, three samples of the dialysate were collected every 15 min and measured for [GABA]o to compare with the [GABA]o in the room air condition. The mixing of N2 gas was then stopped, and room air was flushed abruptly to return FO2 to the room air level. Microdialysis was continued for another 15 min in the room air condition. All studies were conducted under a poikilocapnic condition. This study was performed in nine sham-operated rats and in six CBD rats. In these rats, the ventilation was examined simultaneously with the microdialysis study.

**Group 2: effect of hypoxia on [GABA]o outside of the NTS.**

In six rats (for group 2, n = 6), the guide cannula was implanted outside of the NTS (medullary reticular nucleus) (30). Microdialysis outside of the NTS was performed during hypoxia by use of the same protocol as described above.

**Microinjection Group**

For microinjection group, n = 66.

**Group 3: effects of GABA agonists in the NTS on the early phase of the HVR.** For group 3, n = 24. To examine whether GABA agonists injected into the NTS have a depressant effect on ventilation during the early phase of the HVR, the GABA_A receptor agonist muscimol (n = 6) and the GABA_B receptor agonist baclofen (n = 6) were microinjected into the NTS. After the measurement of baseline ventilation in the room air, 100 nl of each drug or vehicle (PBS) (n = 12) were administered. Ten minutes after the drug injection, the animals were exposed to 10% O2. Ventilation was measured just before hypoxia and after hypoxia.

**Group 4: effects of GABA antagonists in the NTS on the HVD.** For group 4, n = 30. To examine the effects of GABA antagonists on ventilation during the late phase of hypoxia (HVD phase), the GABA_A receptor antagonist bicuculline (10 pmol, n = 6) and the GABA_B receptor antagonists saclofen (400 pmol, n = 6) and CGP (2.5 nmol, n = 6) were microinjected into the NTS. The animal was exposed to 10% O2 for 50 min. At 40 min of hypoxic exposure, 100 nl of each drug or vehicle (PBS) (n = 12) were microinjected. The ventilation was measured just before and after microinjection.

**Group 5: effects of GABA antagonists in the NTS on the ventilation in CBD rats.** For group 5, n = 12. To examine the effects of the GABA antagonists in the NTS on the ventilation in CBD rats (n = 6), bicuculline, saclofen, and CGP were microinjected into the NTS by use the same protocol as for group 4. Each drug was administered on a separate day in random order. To exclude the effects of the operation, six sham-operated rats were also examined in the same way as for group 4. The doses and the volumes of the drugs used in groups 3, 4, and 5 were chosen according to previous articles that dealt with the microinjection of these drugs into rat NTS (6, 20, 37).

**Histological Examination**

The animals were killed by overdose of Nembutal after the experiments. The brain was removed and stored in 10% formalin for 1 day. Serial sections (50 μm) were cut with a vibratome (DTK-1000; Dousaka, Kyoto, Japan) and stained with hematoxylin-eosin for histological confirmation of the location of the microdialysis and microinjection site.

**Data Collection and Analysis**

All data are expressed as means ± SE. The data from the microdialysis study were analyzed by two-way ANOVA with repeated measurement. When significance was indicated, a post hoc Student’s unpaired t-test was used for point-by-point differences between sham-operated rats and CBD rats. The effect of each hypoxic load was analyzed using one-way ANOVA, and a post hoc Scheffe’s test was used to compare baseline and loading periods. The data of the ventilation were obtained as average value of every 1 min for 10 s. We compared the ventilation at the points of 0 min (just before the hypoxic load), 3 min, and 40 min of hypoxia by two-way ANOVA and a post hoc Student’s unpaired t-test for point-by-point differences between sham and CBD rats. The effect of each hypoxic load was also analyzed by use of one-way ANOVA and a post hoc Scheffe’s test. In the GABA agonist study, we also collected ventilation every 1 min for 10 s. We compared the values just before hypoxia and the maximum ventilation induced by hypoxia (usually achieved within 5 min after exposure to hypoxia) with the unpaired t-test. In the GABA antagonist study, the maximum ventilation was usually achieved 1 or 2 min after the drug injection and maintained for at least 5 min. Therefore, we compared the ventilation just before and 2 min after the drug/vehicle injection with the unpaired t-test. The microinjection data obtained from CBD rats and sham-operated rats were analyzed with the paired t-test. Differences between mean values were considered significant when P < 0.05.

**RESULTS**

**Effects of Hypoxia on Ventilation**

The characteristics of baseline respiration across all measured ventilations of rats are summarized in Table 1. The baseline respiration was significantly influenced by CBD (n = 12). Figure 1 shows the changes in minute ventilation, respiratory frequency, and tidal volume during hypoxic (10% O2) exposure in sham-operated rats (n = 9) and CBD rats (n = 6). In sham-operated rats, minute ventilation (Fig. 1A) reached its summit within a few minutes during hypoxic exposure as the early phase of the HVR. It gradually declined and reached the peak stable level between room air ventilation and maximum ventilation as the late phase of the HVR, namely the HVD. Respiratory frequency in-

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<th>Table 1. Characteristics of baseline respiration</th>
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<td><strong>Minute Ventilation, ml/min</strong></td>
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<td>Carotid body intact (n = 69)</td>
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Values are means ± SD. Characteristics of baseline respiration across all measured ventilations of rats. CBD, carotid body denervation. Statistical significance determined by unpaired t-test: NS = not significant.
increased with hypoxia, and this increase in frequency was sustained during the hypoxic exposure (Fig. 1B). Tidal volume also reached its summit, but it gradually declined during the sustained hypoxia (Fig. 1C). Thus the decline of minute ventilation during sustained hypoxia was mainly due to the change in tidal volume. In the CBD rats, minute ventilation before hypoxia was significantly lower than that of the sham-operated rats (Fig. 1A). Because of the small number of CBD rats used in the experiments shown in Fig. 1 (n = 6), there is statistical significance only in minute ventilation. Neither minute ventilation, respiratory frequency, nor tidal volume increased in the CBD rats during hypoxia (Fig. 1, B and C). All of these parameters (minute ventilation, respiratory frequency, and tidal volume) differed significantly between the two groups (P < 0.01, 2-way ANOVA). Both minute ventilation and tidal volume in the sham-operated rats decreased significantly at 40 min of hypoxia compared with those at 3 min of hypoxia (P < 0.01, in both, Scheffé's test).

Effects of GABA Agonist Microinjection on Ventilation

Figure 2 shows the effects of GABA agonist microinjection into the NTS on ventilation during room air breathing and during the early phase of the HVR. In the room air condition, ventilation was not altered by microinjection of the GABA<sub>A</sub> receptor agonist muscimol or the GABA<sub>B</sub> agonist baclofen. However, both GABA<sub>A</sub> and GABA<sub>B</sub> receptor agonists attenuated the increment of ventilation during the early phase of the

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Fig. 1. Changes in minute ventilation, respiratory frequency, and tidal volume during hypoxic (10% O<sub>2</sub>) exposure. Effect of hypoxia (10% O<sub>2</sub>) on minute ventilation (A), respiratory frequency (B), and tidal volume (C) in the sham-operated rats (●; n = 9) and carotid body denervation (CBD) rats (●; n = 6). Values are means ± SE. **P < 0.01 compared with room air (0 min) level, and ‡P < 0.01 compared with 3 min of hypoxia (Scheffé's test). ††P < 0.01 between sham-operated and CBD rats (unpaired t-test).

Fig. 2. Effects of γ-aminobutyric acid (GABA) receptor agonists on the early phase of the hypoxic ventilatory response (HVR). Effects of the GABA<sub>A</sub> receptor agonist muscimol (A) and the GABA<sub>B</sub> receptor agonist baclofen (B) on ventilation in the room air condition and during the early phase of the HVR. For each group, n = 6. Room air, the ventilation in the room air condition 10 min after drug/vehicle microinjection; hypoxia, the maximum ventilation induced by hypoxia. Values are means ± SE. *P < 0.05 and **P < 0.01 compared with the vehicle group (unpaired t-test).
HVR (Fig. 2, top; $P < 0.01$ for muscimol, $P < 0.05$ for baclofen). The increment of respiratory frequency by hypoxia was similar in both GABA agonist and vehicle treatment groups (Fig. 2, middle). However, the increase in tidal volume was significantly reduced by both GABAA and GABA B receptor agonist treatment (Fig. 2, bottom; $P < 0.01$ for muscimol, $P < 0.05$ for baclofen). GABA agonists in the NTS significantly attenuated the early phase of the HVR, and the reduction of minute ventilation was due to the reduction of tidal volume.

Effects of Hypoxia on Extracellular GABA in the NTS

Figure 3 shows the changes in [GABA]o during hypoxic exposure expressed as a percentage of the baseline value. [GABA]o just before hypoxic exposure was adopted as the room air value. [GABA]o in the NTS of the sham-operated rats ($n = 9$) during hypoxic exposure was 61.6 ± 11.8 fmol/μl. It did not increase in the first 15 min of hypoxia. However, in the dialysate of the middle and the last 15 min of hypoxia, [GABA]o increased significantly compared with the room air level (Scheffe’s test, **$P < 0.01$). In the middle (15–30 min) and the last 15 min of hypoxic exposure (30–45 min), [GABA]o in the NTS of the CBD rats and [GABA]o outside of the NTS decreased significantly compared with that of the sham-operated rats ($\dagger P < 0.05$ and $\dagger\dagger P < 0.01$ between [GABA]o in the NTS of sham-operated rats and outside of the NTS; $\dagger P < 0.01$ between sham-operated rats and CBD rats; unpaired $t$-test). Values are means ± SE expressed as percentages of baseline value.

Microinjection of GABA Antagonists During the HVD

For further characterization of GABA in the HVD, GABA receptor antagonists were microinjected into the NTS. The change in ventilation before and after microinjection of the GABA antagonists during the HVD phase (at 40 min of hypoxia) is shown in Fig. 4. The GABA A receptor antagonist bicuculline significantly increased minute ventilation, whereas the vehicle alone did not ($P < 0.01$ compared with the vehicle, unpaired $t$-test; Fig. 4A, top). The two GABA B receptor antagonists, saclofen and CGP, also significantly in-
creased ventilation compared with the vehicle ($P < 0.01$ in both groups, unpaired $t$-test; Fig. 4B, top). Both the GABA$_A$ and the GABA$_B$ receptor antagonists significantly increased tidal volume compared with the vehicle (Fig. 4, A and B, bottom; $P < 0.01$ for both). Neither the GABA$_A$ nor the GABA$_B$ antagonist significantly changed the respiratory frequency (Fig. 4, A and B, middle). GABA antagonists significantly increased the depressed ventilation during the HVD, and this increase in ventilation was mainly due to the increase in tidal volume.

Effect of GABA Antagonists on Ventilation in the CBD Rats During Hypoxia

We also examined the effects of GABA antagonist microinjection into the NTS on ventilation at 40 min of hypoxia in the CBD and sham-operated rats. In the sham-operated rats, GABA antagonist microinjection during this phase significantly increased the depressed ventilation (Fig. 5A; $P < 0.01$, paired $t$-test). In contrast, it did not alter ventilation in the CBD rats (Fig. 5B).

Histological Examination

Figure 6 shows the site where the probe for the microdialysis was placed. Figure 6 is a photomicrograph montage of a coronal section of the medulla caudal to the obex. Figure 6, A–C, shows the probe track in a different animal used in the microdialysis study in the NTS. The probe track was placed within the caudal, intermediate part of the NTS (30), where the carotid sinus nerves terminate (12). Figure 6D shows the probe track in the microdialysis study outside of the NTS. The probe was located in the medullary reticular nucleus (30). The microinjection site was the same as the area shown in Fig. 6, A–C.

DISCUSSION

In this study, we have demonstrated that GABA in the NTS is involved in the HVD in unanesthetized, freely moving rats. The main findings in the present study are as follows. 1) GABA agonists in the NTS had a depressant effect on ventilation during hypoxia. 2) [GABA]$_o$ in the NTS increased during the HVD. 3) GABA antagonists in the NTS increased the depressed ventilation during the HVD. 4) The [GABA]$_o$ increase in the NTS during hypoxia was abolished by CBD. 5) GABA antagonists had no effects on ventilation during hypoxia in the CBD rats.

In our microinjection study, GABA agonists in the NTS reduced ventilation in the early phase of the HVR. There are several reports that GABAergic drugs in the NTS have a depressant effect on ventilation. In anesthetized cats, hypoxic loading after microinjection of muscimol, a GABA$_A$ receptor agonist, into the NTS did not increase the phrenic nerve activity (6). Suzuki et al. (36) reported that in anesthetized rats, microinjection of baclofen into the NTS did not elicit the ventilatory response, even when chemoreceptors were stimulated by intracarotid administration of sodium cyanide. Our results are in agreement with these previous studies and strongly suggest that GABA in the NTS has a depressant effect on ventilation during hypoxia.
We measured GABA in the NTS by an in vivo microdialysis technique. GABA in the NTS significantly increased in the late phase of the hypoxic ventilatory response in the sham-operated rats. However, in the NTS of the CBD rats and outside of the NTS, GABA did not change during hypoxia. These results suggest that the GABA increase during hypoxia is not a nonspecific effect in the CNS and that without chemoreceptor stimulation, GABA does not increase in the NTS even during hypoxia.

To clarify further the role of GABAergic mechanisms in the HVD, we also microinjected GABA antagonists into the NTS. We microinjected these drugs at 40 min of hypoxic exposure because, in this phase, $[\text{GABA}]_o$ in the NTS was significantly increased and the HVD was obvious. Microinjection of the GABA$_A$ receptor antagonist bicuculline and the GABA$_B$ receptor antagonists saclofen and CGP during the HVD phase reversed the depressed ventilation by increasing tidal volume. These results are consistent with previous reports that the HVD is characterized by a decrease in tidal volume (26) and that systemic administration of the GABA antagonists altered the HVD by increasing tidal volume (13, 26). Because these drugs affected only the tidal volume, there is a possibility that the nerves from pulmonary stretch receptors within the NTS were depressed by the increased GABA during hypoxia (19). In contrast, in the CBD rats, the GABA increase during hypoxia was abolished and no increase in ventilation was observed after GABA antagonist microinjection during hypoxia. These results suggest that the increased GABA by peripheral chemoreceptor stimulation has an important role in the ventilatory depression.

In our study, the GABA antagonists did not cause the depressed ventilation to show its full response to hypoxia. Because we used only one dose of each drug, the dose of the GABA antagonists may not have been sufficient for the depressed ventilation to cause a complete recovery from the HVD. Additionally, for technical reasons, we microinjected these drugs unilaterally. There is a possibility that the GABA antagonists may not diffuse throughout the whole area of the NTS. Previous studies show that opioids (8), adenosine (31, 41), nitric oxide (10, 11), and serotonin (31) as well as GABA may be related to the HVD. In our study, we could not rule out the possibility that not only GABAergic mechanisms but also other mechanisms and sites (33) in the CNS may be involved in the HVD. Further studies are needed to clarify these points.

The GABA concentration increased gradually in the NTS during the late phase of hypoxia only in the carotid body-intact rats. The first possible mechanism that may explain these findings is that GABA may be converted mainly from the increased glutamate during hypoxia. In the CNS, GABA is synthesized in a reaction catalyzed by glutamic acid decarboxylase (GAD) and degraded by GABA-α-oxoglutarate transaminase (GABA-T) into succinic semialdehyde (15, 29). It is known that GAD activity increases and GABA-T activity decreases during hypoxia (26). We have demonstrated that glutamate increased in the NTS during the early phase of the HVR according to the peripheral chemoreceptor stimulation (23). In the present study, it was demonstrated that the GABA increase during hypoxia also required peripheral chemoreceptor stimulation. In consideration of these findings, the
gradual increase of GABA during hypoxia may be accounted for as follows. By peripheral chemoreceptor stimulation with hypoxia, glutamate, which stimulates ventilation, increases in the NTS. This increased glutamate is converted into GABA in turn by GAD, which is more activated during hypoxia. Therefore, GABA accumulates in the NTS during the late phase of hypoxia.

Second, it has been reported that there are many GABA-immunoreactive synapses in the NTS and that adjacent GABA-immunoreactive and glutamate-immunoreactive terminals make synaptic contact with the same dendrites within the NTS (3, 32). Thus hypoxia induces glutamate release, and GABAergic interneurons are activated in turn, resulting in GABA accumulation in the NTS. Third, other nuclei may sense hypoxia or neural activity in the NTS and affect the neural activity in the NTS through GABAergic neurons. However, we could not confirm the mechanism of the GABA increase in the NTS in the present study.

Because we used unanesthetized, freely moving rats in this study, there are some technical limitations that should be discussed. First, it was difficult to keep end-tidal partial pressure of CO₂ (PetCO₂) constant during the experiment. Thus it may be argued that the hypocapnia that followed hypoxic hyperventilation could be associated with the HVD in this study. However, we considered that this effect accounts for a small portion of the HVD for the following reasons. In normal humans, the ventilatory response to isocapnic hypoxia is biphasic, and the decline of minute ventilation is not significantly affected by the PetCO₂ levels (35, 41.8, and 44.3 Torr) (9). If hypoxic exposure is prolonged or made more severe, medullary alkalosis induced by the initial hypoxic hyperventilation changes into acidosis, but the respiratory depression is even greater (26). In humans, although jugular venous PCO₂, which is considered to reflect the brain tissue PCO₂, is significantly decreased in the initial phase of hypoxia, it remains unchanged from 5 to 15 min into moderate hypoxia. However, the HVD occurs just during this phase (5–15 min of hypoxia) (35). Additionally, in our study, the fact that the GABA antagonist increased ventilation during the HVD strongly suggests that endogenous GABA in the NTS is related to the HVD. Second, it was difficult to sample arterial blood and expired gas during hypoxia. Thus we could not exclude the metabolic effect of increased GABA in the NTS. Although we could find no previous papers dealing with the metabolic effects of GABA on the respiration in the NTS, Kneussl et al. (18) stated that ventriculo-cisternal perfusion of GABA depresses ventilation with the decrease of O₂ consumption and CO₂ production. Whether GABA in the NTS has metabolic effects on ventilation is not known yet, but there is a possibility that GABA in the NTS changes the systemic metabolism and induces respiratory depression.

We studied both GABA_A and GABA_B receptor-mediated mechanisms in the NTS. However, we could not determine any definite differences in the responses between GABA_A and GABA_B mechanisms. Because high concentrations of saclofen partially suppress GABA_A receptor-mediated mechanisms (25), we used one more GABA_B receptor antagonist, CGP-35348, which may not interact with the GABA_A receptor (28). However, the effects of the GABA_A and GABA_B receptor antagonists and agonists on the ventilation were the same in this study. Both GABA_A and GABA_B antagonists and agonists affected only the tidal volume, not the respiratory frequency, during hypoxia. We have no ready explanation for why both GABA_A and GABA_B-mediated mechanisms had the same effects on the ventilation during hypoxia. However, there is a report showing that intravenously injected GABA_A receptor agonist (muscimol) decreased tidal volume, whereas GABA_B receptor agonist (baclofen) decreased the respiratory frequency (7), therefore differing from our results. There is a possibility that these GABAergic drugs may not be purely selective to each type of receptor at the doses used in our study. In addition, we microinjected these drugs into the NTS. However, the effect of these GABAergic drugs on ventilation may be different in other sites of the CNS. Further studies are needed to clarify this point.

In summary, we have examined the role of the GABAergic mechanism in the NTS during the HVD in unanesthetized, freely moving rats. It has been demonstrated that the [GABA]₀ in the NTS increases during the HVD and that the depressed ventilation is increased by GABA antagonist microinjection. The increase in GABA during the HVD is abolished by CBD, and the GABA antagonists had no effect on the ventilation in the CBD rats during hypoxia. Therefore, this study indicates that the GABAergic mechanism in the NTS has a pivotal role in the HVD and may be actually functioning in awake animals. Chemoreceptor stimulation may be essential to activate the GABAergic mechanism in the NTS.

Perspectives

In this study, we have demonstrated that GABA in the NTS has an important role in the HVD. There are many previous studies that examined the mechanisms of the HVD, but many aspects of the HVD remain unclear. First, for example, the neuronal connections that convey the inhibitory signal have not been elucidated. Thus clarification of these neuronal mechanisms would help us to understand the mechanisms of respiration and the neuronal networks involved under a hypoxic condition. Second, the physiological role of the HVD is still unclear. Respiratory depression seems to worsen hypoxemia when the supply of O₂ is limited. However, for the cellular adaptation to hypoxia, it has been suggested that the depression of cellular activity is an important example of hypoxia-adaptive mechanisms (26). This strategy may relate to the hypoxic tolerance of the cells and organs, and the HVD would seem to play an important role in this adaptation. Third, the HVD seems to be related to clinically important problems. It was reported that the patients who had a history of near-miss death from sudden infant death syndrome (14) and asthma attacks (16) have blunted hypoxic chemosensitivity. It thus seems possible that the HVD plays a role in the causes of deaths
in these patients. Therefore, examination and clarification of the mechanisms of the HVD will increase our understanding of physiologically and clinically important issues such as the hypoxic tolerance when the supply of O₂ is limited.

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