Stress-induced attenuation of the hypercapnic ventilatory response in awake rats

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Kinkead, Richard, Lydie Dupenloup, Nadine Valois, and Roumiana Gulemetova. Stress-induced attenuation of the hypercapnic ventilatory response in awake rats. J Appl Physiol 90: 1729–1735, 2001.—To test the hypothesis that stress alters the performance of the respiratory control system, we compared the acute (20 min) responses to moderate hypoxia and hypercapnia of rats previously subjected to immobilization stress (90 min/day) with responses of control animals. Ventilatory measurements were performed on awake rats using whole body plethysmography. Under baseline conditions, there were no differences in minute ventilation between stressed and unstressed groups. Rats previously exposed to immobilization stress had a 45% lower ventilatory response to hypercapnia (inspiratory CO$_2$ fraction = 0.05) than controls. In contrast, stress exposure had no statistically significant effect on the ventilatory response to hypoxia (inspiratory O$_2$ fraction = 0.12). Stress-induced attenuation of the hypercapnic response was associated with reduced tidal volume and inspiratory flow increases; the frequency and timing components of the response were not different between groups. We conclude that previous exposure to a stressful condition that does not constitute a direct challenge to respiratory homeostasis can elicit persistent (≥24 h) functional plasticity in the ventilatory control system.

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though these data suggest that exposure to a stressful situation that is not directly relevant to respiration could modify the performance of the respiratory control system, the functional consequences of processive stress exposure on the respiratory control system have not been addressed. To test the hypothesis that immobilization stress elicits plasticity in the respiratory control system, we compared the hypoxic and hypercapnic ventilatory responses of awake, unrestrained rats previously subjected to daily psychological stress sessions (immobilization stress for 90 min/day) with those of routinely handled animals.

MATERIALS AND METHODS

Experiments were performed on 70 adult male Sprague-Dawley rats (357 ± 8 g; Charles River Canada, St-Constant, PQ, Canada). Rats were supplied with food and water ad libitum and maintained in standard laboratory conditions (20°C, 12:12-h dark-light cycle; lights on at 0600 and off at 1800). To address the effects of processive (i.e., psychological, nonsystemic) stress on ventilatory control, the study involved two series of ventilatory measurements (hypoxia and hypercapnia) that were performed 24 h after the end of the immobilization stress protocol. In each series, three groups of animals were studied, and each group received a different number of daily stress sessions before ventilatory measurements. All experiments were performed according to the guidelines of the Canadian Council on Animal Care. The institutional animal care committee approved the specific protocols.

Experimental groups and protocol summary. Series I tested the effects of immobilization stress on the hypoxic ventilatory response [inspiratory O2 fraction (FiO2) = 0.12]; series II addressed the effects of immobilization stress on the ventilatory response to hypercapnia (FiCO2 = 0.05). Each series involved three groups: nonstressed control rats (n = 20 and 16 for series I and II, respectively) and rats exposed to one (n = 5 for both series I and II) or two (n = 11 and 13 for series I and II, respectively) daily immobilization stress sessions.

Ventilatory responses to hypoxia or hypercapnia were measured by whole body plethysmography 24 h after the last stress session. In some animals in each group, a chronic indwelling arterial catheter was placed for analysis of arterial blood gases. Ventilatory responses of rats in which blood samples were taken were not different from those of nonsampled animals (P = 0.53 and 0.57 for series I and II, respectively).

Immobilization stress protocol. An immobilization stress session consisted of placing the rat in a Broome rodent restrainer for 90 min. For each series, one group of rats was exposed to a single stress session and a second group was subjected to the stress protocol on 2 consecutive days. The stress protocol was always performed between 0900 and 1200. For each series (hypoxic and hypercapnic), the corresponding control group consisted of rats that were not subjected to the stress protocol.

Surgical procedure. A catheter was placed in the femoral artery of rats for blood sampling and measurement of arterial blood gases and pH. Arterial catheters were placed under isoflurane anesthesia (2–2.5% in O2). Once in position, the catheter was routed under the skin to the back of the neck and filled with heparinized saline (10 U/ml). Postsurgical care consisted of two subcutaneous injections of an anti-inflammatory drug (ketoprofen, 2 mg/kg): one immediately after the surgery and another 24 h later. The second catheter was flushed once daily with heparinized saline to ensure patency. Rats recovered for ≥48 h before ventilatory measurements were made.

Measurements of ventilation and arterial blood gases. Ventilation of tethered (with catheters only), but otherwise unrestrained, unnanesthetized rats was measured using a whole body, flow-through plethysmograph (model PLY3223, Buxco Electronics, Sharon, CT). The system was similar to that described by Hamelmann et al. (16) and consisted of a 4.5-liter Plexiglas experimental chamber equipped with two pneumotachographs with a defined resistance. Differential pressure between the experimental and reference chambers was measured with a differential pressure transducer (SenSym) with a fast response time (500 µs). The pressure signal was amplified and then integrated by data analysis software (Buxco Biosystem XA). The system was calibrated by injecting a known volume (1 ml) into the chamber with a glass syringe. The barometric pressure and rat body weight were recorded daily, and the chamber temperature and humidity and core temperature of the animal were measured at the beginning and end of each experimental period. These data were used to express tidal volume (VT) in milliliters (BTPS) per 100 g. Fresh air or gas mixtures were delivered to the experimental chamber at a constant rate with a bias flow regulator (model PLY1020, Buxco Electronics). The gas mixture flowing out of the chamber was analyzed with a flow-through capnograph (Novametrix, Wallingford, CT) for subsequent calculation of CO2 production (V˙CO2) with an open system according to the method and equations described by Mortola and Dotta (32). CO2 measurements from the outflowing gas mixture also ensured that CO2 levels within the chamber always remained below 0.5%. Typical airflow ranged between 2.0 and 2.5 l/min.

After rectal (core) temperature was recorded, the rat was placed in the box with room air flowing through. When necessary, the arterial catheter was connected to the swivel for blood sampling. The animal would typically explore the surroundings, groom itself, and then settle down. Baseline measurements were made when the animal was quiet but awake and breathing room air. A first arterial blood sample of 100 µl was taken at that time. Arterial blood samples were obtained by slowly drawing enough blood (∼0.3 ml) to ensure that the blood within the catheter was not diluted with saline. The catheter was then disconnected from its extension near the experimental chamber and placed into a small heparinized glass capillary that fitted tightly around the catheter, thus avoiding exposure of the blood to room air. Blood would then rapidly flow within the capillary (due to blood pressure and/or gravity pull), and the sample was analyzed immediately. Then a gas mixture of 12% O2 or 5% CO2 in air was delivered to the chamber for 20 min, and a second arterial blood sample was taken before the recording chamber was opened for a final body temperature measurement. Blood samples were analyzed for arterial PO2 (PaO2), PCO2 (PaCO2), and pH (AVL model 995). In each series, animals were exposed to only one respiratory stimulus. All measurements were performed between 1000 and 1500.

Data analysis. Baseline measurements of ventilatory variables were obtained by averaging 10 min of stable recording, whereas a 5-min average was taken for each variable at the end of the hypoxic or hypercapnic exposure. The results were analyzed statistically using a two-way ANOVA (Statview 5.0, SAS Institute, Cary, NC) followed by a post hoc Fisher's protected least significant difference test (P < 0.05). A repeated-measure design was used when appropriate.
RESULTS

Immobilization stress and “resting” ventilation. Baseline ventilatory measurements obtained in both series of experiments were comparable to those reported in other studies using Sprague-Dawley rats under similar experimental conditions (8, 12, 13, 34, 36). Exposure to one or two stress sessions did not have any statistically significant effect on any of the ventilatory variables measured under normoxic normocapnic conditions (Tables 1 and 2; Figs. 1 and 2). However, both groups of stressed rats had slightly greater V̇ CO 2 and PaCO 2 values than control animals (Tables 1 and 2). Immobilization stress and hypoxic ventilatory response. In this series, isocapnia was not maintained during hypoxia; yet, PaO 2 and PaCO 2 were reduced equally in all three groups. Ventilatory measurements obtained at the end of the hypoxic stimulus revealed no difference between stressed and control rats (Fig. 1, Table 1). Similarly, hypoxic values for the ratio of V̇ T to inspiratory time (V̇ T/I), an index of inspiratory effort, were not different between stressed and unstressed rats (Fig. 1D). Mean V̇ O 2 was unaffected during the first 20 min of hypoxia in all groups (Table 1), in agreement with previous reports (26).

To further analyze the effects of stress on the hypoxic response, selected ventilatory variables were normalized and expressed as a percent change from baseline values. Although this additional analysis suggests that exposure to two stress sessions may attenuate the hypoxic increase in minute ventilation, this observation was not statistically significant (Fig. 3).

Table 2. Effects of stress on ventilatory variables, arterial blood gases, and CO 2 production under normoxic (baseline) and hypercapnic conditions in awake rats: series II

<table>
<thead>
<tr>
<th>P Value</th>
<th>Stress effect</th>
<th>Hypocapnic effect</th>
<th>Stress × hypocapnic interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.979</td>
<td>&lt;0.0001</td>
<td>0.638</td>
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<tr>
<td>0.940</td>
<td>&lt;0.0001</td>
<td>0.970</td>
<td></td>
</tr>
<tr>
<td>0.622</td>
<td>&lt;0.0001</td>
<td>0.514</td>
<td></td>
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</tbody>
</table>

Values are means ± SE. Baseline values were obtained in quiet but awake rats ≥1 h after the animal acclimated to the plethysmographic chamber. Hypercapnic values were obtained after 20 min of exposure to hypoxic (inspired O 2 fraction = 0.20). Arterial blood gases were not obtained from all animals; the number of samples taken for each group is given in parentheses. Boldface indicates statistically significant effect of stress or hypoxia (P < 0.05). * Statistically different from baseline (P < 0.05). † Statistically different from corresponding control (P < 0.05).
Immobilization stress and hypercapnic ventilatory response. Exposure to moderate hypercapnia had a significant effect on all variables reported in Table 2. Specifically, inspiratory and expiratory duration were shortened, $\text{PaO}_2$ and $\text{PaCO}_2$ increased, and arterial pH and body temperature decreased. Changes in $\text{PaO}_2$ and $\text{PaCO}_2$ were not different between experimental groups. These responses to hypercapnia were not affected by previous stress exposure, as indicated by the lack of statistical interaction between the two factors. $\dot{VCO}_2$ was reduced (Table 2), in accordance with the study of Ling et al. (26). Expressing hypercapnia-induced changes in $\dot{VCO}_2$ as a percent change from baseline revealed no difference between stressed and unstressed rats ($P = 0.345$; data not shown).

Exposure to a single immobilization stress session was sufficient to attenuate the hypercapnic ventilatory response. This effect of immobilization stress was also detected in rats exposed to two stress sessions (Fig. 2). Reduced responsiveness to hypercapnic stimulation was not related to the frequency component of the response; neither the breathing frequency nor the timing of the breathing cycle was affected by immobilization stress (Fig. 2A, Table 2). In contrast, the increase in $V_t$ during hypercapnia was less in both groups of rats exposed to immobilization stress (Fig. 2B) owing to a lower increase in inspiratory effort as indicated by $V_t/T_l$ calculations (Fig. 2D). Statistical analysis revealed significant interactions between stress and hypercapnia on minute ventilation, $V_t$, and $V_t/T_l$, indicating that stress exposure significantly reduced the ability to respond to hypercapnia ($P = 0.032, 0.013, \text{and } 0.030$, respectively).

Again, expressing the hypercapnic ventilatory response in terms of percent change from baseline values confirmed that stress exposure was associated with a reduced responsiveness to hypercapnia. Figure 3B shows that the increase in minute ventilation during hypercapnia was less in both groups of rats subjected to our immobilization stress protocol, owing mainly to a reduced $V_t$ response and $V_t/T_l$. Immobilization stress did not affect the frequency component of the response ($P = 0.641$).

**DISCUSSION**

Collectively, our results indicate that exposure to a single session of processive (i.e., nonsystemic) stress is sufficient to alter the responsiveness to moderate hypercapnia in awake rats, even though immobilization stress does not constitute a direct challenge to respiratory homeostasis. This manifestation of respiratory plasticity, which persisted for $\geq 24$ h after the last stress session ended, may be unique to the neural circuits involved in the ventilatory response to hypercapnia, because immobilization stress did not affect the...
hypoxic ventilatory response. Our results are consistent with our working hypothesis that exposure to pro cessive stress can elicit persistent functional plasticity of neural circuits involved in ventilatory control.

**Stress and ventilatory response to hypercapnia.** In both series of experiments, V̇CO₂ and PaCO₂ values were elevated in stressed rats vs. control animals. Although the increase in V̇CO₂ is in accordance with other reports suggesting that restraint stress increases metabolism (23, 33), the lack of significant differences in resting minute ventilation between groups is consistent with a potential reduction in the resting hypercapnic ventilatory drive in stressed rats. These results are in agreement with plethysmographic measurements showing that previous exposure to a single immobilization stress session is sufficient to attenuate the ventilatory response to moderate hypercapnia in awake rats. Previous stress exposure did not affect the timing and frequency component of the hypercapnic response but reduced the magnitude by which VT/Ti and VT increased during hypercapnia, thus indicating that the stress-induced attenuation of the hypercapnic ventilatory response was related to a reduction in inspiratory effort.

Isom and Elshowihy (20) reported that rats exposed to inescapable foot shock displayed an increase in respiratory rate, VT, and minute ventilation and that systemic pretreatment with the opioid receptor antagonist naloxone hydrochloride potentiated this response. Their study also showed that the hypercapnic ventilatory response was attenuated after chronic exposure (11 daily sessions) to this stress paradigm. On the basis of these results, these authors concluded that endogenous opioids prevent excessive stimulation of respiration by stress. Several important differences in experimental protocol prevent us from extending this conclusion to our results. For instance, Isom and Elshowihy measured ventilatory responses immediately after stress exposure, whereas our measurements were performed 24 h after the last stress session. Moreover, unlike immobilization stress, foot shock is a nociceptive stimulus, induces analgesia (15, 35), and is thus more likely to induce opioid release. These differences do not eliminate the possibility that opioids are involved in stress-induced respiratory plasticity but prevent us from eliminating the potential contribution of other neural mechanisms.

The fact that immobilization stress affected only the hypercapnic ventilatory response suggests, albeit indirectly, that previous stress exposure affected neural pathways involved in the hypercapnic, but not the hypoxic, response. Recent neuroanatomic data provided by Berquin et al. (4) are consistent with this idea. These authors mapped neuronal populations expressing the protein Fos after exposure to moderate hypoxia (FIO₂ = 0.11) or hypercapnia (FICO₂ = 0.05). Their results revealed important differences in the pattern of Fos expression between the two ventilatory stimuli. A key feature is the demonstration that hypercapnia, but not hypoxia, increased Fos immunoreactivity in the locus coeruleus (LC) and the paraventricular nucleus of the hypothalamus (PVH), two structures that play critical roles in the coordination of the stress response (for review see Refs. 11 and 18). Local acidification of noradrenergic neurons of the LC increases respiratory frequency and phrenic nerve discharge in cats (10). Moreover, intrinsic activity of LC neurons is exquisitely sensitive to hypercapnia (37), and exposure to CO₂ increases Fos immunoreactivity in several brain stem noradrenergic regions, including the LC (4, 17, 40). Noradrenergic LC neurons therefore appear to be an important part of the hypercapnic ventilatory response (17). Although the role of the PVH in ventilatory control is less documented, neuroanatomic tracing studies suggest a direct connection between the PVH and phrenic (inspiratory) motoneurons (42). Moreover, the same study showed that chemical activation of the PVH increases diaphragmatic electromyogram activity (42). Nonetheless, the sum of these data raises the possibility that the LC and PVH may be part of a parallel pathway in the neural control of breathing that acts at the interface between systemic (chemosen-
sory) and processive (psychological) influences on respiratory motor behavior.

**Stress and the hypoxic ventilatory response.** Whether expressed as absolute values or percent change from baseline, none of the ventilatory variables measured under the hypoxic condition were significantly affected by previous stress exposure. We have retained three possible explanations for the lack of stress-induced changes in the short-term hypoxic ventilatory response. First, a stronger hypoxic stimulus may have revealed differences in the hypoxic ventilatory response between stressed and unstressed rats; however, severe hypoxia (i.e., PaO₂ < 40 Torr) would raise questions concerning the physiological significance of stress-induced respiratory plasticity. Second, immobilization stress did not affect carotid body function and, unlike hypercapnia, stress-induced neural plasticity occurred in regions that are not directly relevant to the short-term hypoxic ventilatory response. Finally, because stress affects the serotonergic system (9), immobilization stress may affect other time domains of the hypoxic ventilatory response such as long-term facilitation, a serotonin-dependent manifestation of respiratory plasticity (14, 38). This hypothesis remains to be tested.

**Perspectives**

Our results showed that exposure to immobilization, a processive stress, attenuates the responsiveness to hypercapnia in rats. This effect was observed after exposure to a relatively mild stress paradigm and a moderate hypercapnic stimulus in an awake animal. These findings have important potential implications to many studies where various forms of stress (including immobilization) may be an inherent part of the experimental protocol. Yet the potential implications of stress in assessment of ventilatory control is often dismissed by most investigators.

The effects of previous stress exposure on ventilation are likely to be more notable when respiratory drive is further reduced, such as during sleep or anesthesia. This hypothesis is consistent with the irregular nocturnal breathing pattern observed in patients suffering from stress-related neurological diseases, such as panic disorders, which also show an increased rate of apneas compared with healthy subjects (39). The functional significance of stress-induced attenuation of hypercapnic responsiveness as a manifestation of respiratory plasticity remains unclear but may be part of a more general strategy aimed at attenuating the deleterious effects of stress.

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Some of these results have been reported in abstract form (21).

**REFERENCES**


