Depressed ventilatory response to hypoxia in hypothermic newborn piglets: role of glutamate

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Mc Cormick, Annette, Cleide Suguihara, Jian Huang, Carlos Devia, Dorothy Hehre, Jocelyn H. Bruce, and Eduardo Bancalari. Depressed ventilatory response to hypoxia in hypothermic newborn piglets: role of glutamate. J. Appl. Physiol. 84(3): 830–836, 1998.—To evaluate whether changes in extracellular glutamate (Glu) levels in the central nervous system could explain the depressed hypoventilatory response in hypothermic neonates, 12 anesthetized, paralyzed, and mechanically ventilated piglets <7 days old were studied. The Glu levels in the nucleus tractus solitarius obtained by microdialysis, minute phrenic output (MPO), O₂ consumption, arterial blood pressure, heart rate, and arterial blood gases were measured in room air and during 15-min periods of isocapnic hypoxia (inspired O₂ fraction = 0.10) at brain temperatures of 39.0 ± 0.5°C (normothermia [NT]) and 35.0 ± 0.5°C (hypothermia [HT]). During NT, MPO increased significantly during hypoxia and remained above baseline. However, during HT, there was a marked decrease in MPO during hypoxia (NT vs. HT, P < 0.03). Glu levels increased significantly in hypoxia during NT; however, this increase was eliminated during HT (P < 0.02). A significant linear correlation was observed between the changes in MPO and Glu levels during hypoxia (r = 0.61, P < 0.0001). Changes in pH, arterial PO₂, O₂ consumption, arterial blood pressure, and heart rate during hypoxia were not different between the NT and HT groups. These results suggest that the depressed ventilatory response to hypoxia observed during HT is centrally mediated and in part related to a decrease in Glu concentration in the nucleus tractus solitarius.

shortly after birth infants are particularly vulnerable to hypoxia and hypothermia (HT). Newborn infants exposed to HT environmental conditions exhibit a marked decrease in the ventilatory response to hypoxia (1, 3, 6), but the mechanism responsible for this decrease in ventilation has not been elucidated. A possible explanation for this depressed ventilatory response to hypoxia is the decrease in the metabolic rate that has been shown in newborn kittens exposed to low environmental temperature compared with those in a thermoneutral environment (27). However, more recently, various neuromodulators, such as adenosine, dopamine, endorphins, and amino acid neurotransmitters, have been suggested as possible mediators for the hypoxic ventilatory depression (13, 18, 19, 26, 34). The ventilatory response to hypoxia appears to be determined by the balance of excitatory amino acid neurotransmitters such as glutamate (Glu) and aspartate and inhibitory amino acids such as γ-aminobutyric acid (GABA). In support of this hypothesis, central administration of L-Glu resulted in a marked increase in basal ventilation in adult animals (5, 9), whereas administration of N-methyl-D-aspartate (NMDA) receptor blocker resulted in significant depression of the ventilatory response to hypoxia in unanesthetized piglets (21).

Pastuszko (31) demonstrated a significant increase in extracellular levels of Glu and aspartate in the striatum of newborn piglets exposed to different levels of hypoxia. Several studies in adult animals have explored the role of neurotransmitters in HT conditions associated with ischemia. It has been shown that brain ischemia is associated with increased extracellular levels of excitatory amino acids in the striatum and that HT significantly decreases or completely inhibits the release of these neurotransmitters (2). Adult rats in which brain temperature was maintained at 36°C exhibited a significant increase in Glu levels in the striatum during cerebral ischemia. In contrast, the release of Glu was completely inhibited when brain temperature was lowered to 30–33°C (2).

To our knowledge no studies have linked the effects of HT and the central release of amino acid neurotransmitters during hypoxia on the respiratory control mechanisms in newborns. The present study was designed to test the hypothesis that HT depresses the ventilatory response to hypoxia in the newborn piglet and that this depression is due to blunting of the central release of the excitatory amino acid Glu. The objectives of the study were to define the effect of HT on the ventilatory response to hypoxia in newborn piglets and to measure the concentration of Glu in the region of the nucleus tractus solitarius (NTS) during normoxia and hypoxia under normothermic (NT) and HT conditions.

MATERIALS AND METHODS

Experiments were performed on 12 newborn Yorkshire piglets (1.9 ± 0.3 kg body wt, 4 ± 1 days of age). The animals were randomly assigned to start in NT or HT conditions with a crossover design. The piglets were anesthetized with an intraperitoneal injection of urethane (250 mg/kg) and α-chloralose (40 mg/kg). Femoral arteries and one femoral vein were cannulated and used for systemic arterial blood pressure (ABP) and heart rate (HR) measurements, blood sampling, and infusion of fluids. A tracheostomy was performed, and a 4.0-mm endotracheal tube was inserted. Animals received a continuous infusion of 5% dextrose solution (6 ml·kg⁻¹·h⁻¹) through the peripheral vein. ABP was measured with a pressure transducer (model P-2310, Gould Instruments, Cleveland, OH) and amplified by Gould amplifiers.

Phrenic nerve output. The phrenic nerve was dissected from the C₅ root to the thorax and cut distally. The nerve was desheathed, placed on a platinum bipolar electrode, and...
preserved with a petroleum jelly paste. The phrenic nerve activity was amplified (model S75-05, Coulbourn Instruments, Lehigh Valley, PA) and filtered through adjustable band-pass filters (48 dB/octave, model S75-34, Coulbourn Instruments) at a bandwidth of 30–2,500 Hz for digital collection in a microprocessor at a sampling frequency of 5,000 Hz. Real-time analog-to-digital conversion of phrenic nerve activity was done by a high-speed 12-bit I/O board (model DT2821, Data Translation, Marlboro, MA) at a frequency of 100 Hz. Every burst of the phrenic nerve was identified and validated by the program, which output a filtered and amplified signal that triggered the modified ventilator at a frequency of 100 Hz. The filtered and amplified phrenic nerve signal was then rectified and averaged by a third-order Paynter filter and a 100-ms time constant. Minute phrenic output (MPO) was expressed by the total electrical activity (area under the curve) multiplied by the nerve burst frequency. The phrenic nerve activity was analyzed over a 1-min collection period or a minimum of 10 breaths.

A computer program was developed to control the timing of the time-cycled, pressure-limited ventilator (model IV-100B, Sechrist, Anaheim, CA) to avoid interference of mechanical breaths with the phrenic nerve output. The phrenic nerve signal was band-pass filtered (30–2,500 Hz), rectified, passed through a moving time averager, filtered with a time constant of 200 ms, and amplified. This processed signal was acquired by a computer program by means of an analog-to-digital, digital-to-analog converter (AT-CODAS, Dataq Instruments) at a frequency of 100 Hz. Every burst of the phrenic nerve was identified and validated by the program, which output a control signal that triggered the modified ventilator at a preset time at the end of each phrenic nerve burst.

O₂ consumption measurements. O₂ consumption (V\textsubscript{O₂}) was measured by sampling the expired gas from the side port of the expiratory line of the ventilator circuit (33). The difference between inspiratory and expiratory O₂ concentration was continuously measured with an O₂ analyzer (model 570-A, Servomex, Crowborough, Sussex, UK) throughout the study period. V\textsubscript{O₂} was calculated by the following formula:

\[
V\textsubscript{O₂} = V\textsubscript{S}(F\textsubscript{IO₂} - F\textsubscript{EO₂}),
\]

where $V_S$ is the flow rate through the system, $F\text{IO}_2$ is the fraction of inspired O₂ concentration, and $F\text{EO}_2$ is the fraction of O₂ in mixed expired gas. The flow rate through the system was measured before each run by a linear mass flowmeter (0.0–20.0 l/min; model 8100, Matheson Gas Products, Secaucus, NJ).

Brain stem microdialysis procedure. At the completion of surgery, the animals were paralyzed with pancuronium bromide (0.2 mg·kg\textsuperscript{-1}·h\textsuperscript{-1}) and mechanically ventilated with room air. Each piglet was placed in the prone position with its head fixed in a stereotaxic holder (model 1530, David Kopf, Tujunga, CA). To visualize the atlantooccipital membrane, an incision was made along the midline of the scalp and extended to the occipital region. After the skull was exposed and the neck muscles were retracted, a 1-mm-diameter opening was made in the atlantooccipital membrane, 2.5 mm left of the center point at the horizontal line between the medial end of right and left sutures between the supraoccipital and exoccipital bones. A customized probe holder was used to secure the microdialysis probe at an 84° angle. The dura was perforated with a 27-gauge needle, and the probe was introduced to a depth of 12.5 mm from the external membrane. The measurements used for the placement of the probe were determined during pilot studies.

Through the probe (20 mm shaft length, 2 mm membrane length, 0.5 mm diameter; model CMA/10, CMA, Acton, MA), an unbuffered Ringer solution that contained (mM) 140 NaCl, 2.5 KCl, 1.3 CaCl\textsubscript{2}, and 0.9 MgCl\textsubscript{2}·6H\textsubscript{2}O was perfused continuously via an infusion pump (CMA/102 microdialysis AB pump) at a rate of 2.0 µl/min. The dialysate was collected at 5-min intervals after a stabilization period of 1.5 h. Glu concentration of the dialysate was shown to be stable after the 1.5-h stabilization period by determination of repeated Glu levels. The samples were frozen and stored at −70°C and later analyzed for Glu concentration. At the end of the studies, a fast green dye (5%) was perfused through the microdialysis probe for the determination of the probe position, which was verified by histological studies by a neuropathologist. The probe was considered to be in the area of the NTS if any portion of the distal 2 mm of the probe was inside the NTS. The 11 animals in which the microdialysis probe was confirmed to be in the area of the NTS were used for data analysis (Fig. 1). The samples from one animal could not be analyzed because of technical problems.

Glu analysis. Glu concentrations in the perfusate were analyzed by high-performance liquid chromatography with electrochemical detection after precolumn derivatization. The stable o-phthaldehyde and β-butyrylthiol derivatives of amino acids were separated by gradient elution. The substitution of β-butyrylthiol for 2-mercaptoethanol improved the derivatization stability. The mobile phase for determination of the amino acid content consisted of solvent A (67.8:27.4:4.7) and solvent B (15:45:40); acetate-acetonitrile-tetrahydrofuran (by volume; pH 6.8). The gradient from 0 to 85% was run in 30 min at the flow rate of 0.8 ml/min. The retention time for Glu was 4.0. All work was done on a liquid chromatograph (model BAS 200, Bioanalytical Systems, West Lafayette, IN) equipped for gradient operation and amperometric detection. Amino acids were separated on a Bioanalytical Systems microbore 0.25 column (100 × 1 mm, 3 µm particle diameter); the glassy carbon electrode was maintained at 0.7 V immersed in Ag-AgCl. In vitro recovery of the microdialysis probe to determine efficiency of the probe for Glu was done before and after each experiment.

Induction of HT. A temperature probe was placed in the epidural space to measure brain temperature. The animal's body temperature was maintained by a servo-controlled water bath and water-heated blanket to obtain a brain temperature of 39 ± 0.5°C (NT) or 35 ± 0.5°C (HT). The rectal and brain temperatures were continuously monitored during the study.

Study protocol. The animals were allowed to stabilize for 90 min after the placement of the microdialysis probe. After the stabilization period, measurements of ABP, HR, V\textsubscript{O₂}, arterial blood gases, and phrenic nerve activity were obtained and referred to as room air baseline measurements. Phrenic nerve activity, end-tidal CO₂, ABP, and HR were monitored continuously. The end-tidal CO₂ was kept constant during the study period by adjusting the ventilator rate or pressure. Microdialysis samples were obtained continuously over 5-min intervals for 30 min in room air. Hypoxia was induced by reducing the $F\text{IO}_2$ to 0.10 using an O₂-balance N₂ mixture, and all measurements were repeated at 1, 5, 10, and 15 min. After all measurements were obtained, the animals were returned to room air. If the animal began the study with the NT run, this was followed by an HT run or vice versa.

Handling and care of the animals were in accordance with the guidelines of the National Institutes of Health, and the study protocol was approved by the Animal Care Committee of the University of Miami.

Statistical analysis. Values are means ± SE. Analysis of variance (ANOVA) with repeated measures was used to compare the changes in cardiorespiratory and Glu values...
during hypoxia between the NT and HT groups. Paired t-test was used to compare these variables within the group, and Bonferroni’s correction was used for multiple comparisons. The correlation between changes in MPO and Glu levels during hypoxia was evaluated by a linear regression analysis. P < 0.05 was considered statistically significant.

RESULTS

Ventilatory response to hypoxia. The MPO values obtained during normoxia and hypoxia are presented in Fig. 2. A biphasic ventilatory response to hypoxia was observed in the NT animals. In NT mean MPO increased significantly by 19 ± 5% during the 1st min of hypoxia. This was followed by a slight decrease to 9 ± 12% above baseline at 15 min of hypoxia. In contrast, during HT the initial increase in MPO was eliminated, and this was followed by a significant decrease to 25 ± 7% below baseline values at 15 min of hypoxia (NT vs. HT, P < 0.03, by ANOVA).

Arterial blood gases and acid-base status. Arterial blood gases and acid-base status in room air and after 15 min of hypoxia in NT and HT are shown in Table 1. The arterial Pco2 was maintained constant during normoxia and hypoxia in both groups. Changes in pH, arterial Po2, and base excess with hypoxia were not different between groups.

VO2. Mean values for Vo2 in NT and HT are shown in Fig. 3. Mean Vo2 decreased significantly with hypoxia in both groups: from 10.5 ± 0.6 to 7.2 ± 0.7 ml·min⁻¹·kg⁻¹ in NT (P < 0.01) and from 8.5 ± 0.4 to 5.8 ± 0.4 ml·min⁻¹·kg⁻¹ in HT (P < 0.01, by paired t-test). Although there was a significant decrease in baseline Vo2 from NT to HT (P < 0.02, by ANOVA), the changes
in \( \dot{V}O_2 \) with hypoxia were similar under the two experimental conditions: \(-32 \pm 4\% \) in NT and \(-31 \pm 5\% \) in HT.

ABP and HR. Table 1 shows the ABP and HR during normoxia and after 15 min of hypoxia in NT and HT. There was no significant difference between groups with respect to the changes secondary to hypoxia. The baseline HR values decreased significantly after the animals were exposed to HT \( (P < 0.05, \text{by ANOVA}) \).

Glu changes in the central nervous system in response to hypoxia. Figure 4 shows the changes in Glu concentration in the NTS with hypoxia expressed as percentage of room air. During NT the Glu levels increased by twofold during the first 5 min of hypoxia. This was followed by a decline over time, but Glu levels remained above baseline values. In contrast, the increase in Glu concentration with hypoxia was completely eliminated during HT.

A significant linear correlation was observed between the changes in MPO and NTS Glu levels obtained during 5–10 and 10–15 min of hypoxia \( (r = 0.61, P < 0.001; \text{Fig. 5}) \).

Table 1. Arterial blood gases, acid-base balance, and cardiorespiratory measurements obtained in room air and after 15 min of hypoxia during NT and HT

<table>
<thead>
<tr>
<th></th>
<th>NT</th>
<th>HT</th>
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<tbody>
<tr>
<td></td>
<td>Room air</td>
<td>10% ( O_2 )</td>
</tr>
<tr>
<td>( pH )</td>
<td>7.42 ± 0.01</td>
<td>7.35 ± 0.02*</td>
</tr>
<tr>
<td>( P_{CO_2}, \text{Torr} )</td>
<td>40 ± 1</td>
<td>40 ± 1</td>
</tr>
<tr>
<td>( P_{O_2}, \text{Torr} )</td>
<td>87 ± 4</td>
<td>31 ± 1*</td>
</tr>
<tr>
<td>( BE, \text{mmol/l} )</td>
<td>2.2 ± 1.1</td>
<td>-2.6 ± 1.3*</td>
</tr>
<tr>
<td>( ABP, \text{mmHg} )</td>
<td>78 ± 3</td>
<td>68 ± 3*</td>
</tr>
<tr>
<td>( HR, \text{beats/min} )</td>
<td>283 ± 7</td>
<td>276 ± 6</td>
</tr>
<tr>
<td>( \text{Burst frequency, burst/min} )</td>
<td>41 ± 8</td>
<td>42 ± 8</td>
</tr>
<tr>
<td>( \text{Area activity, au} )</td>
<td>0.014 ± 0.003</td>
<td>0.015 ± 0.004</td>
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</table>

Values are means ± SE. NT, normothermia; HT, hypothermia; \( P_{CO_2} \) and \( P_{O_2} \), arterial \( PCO_2 \) and \( PO_2 \); BE, base excess; ABP, arterial blood pressure; HR, heart rate; au, arbitrary unit. *\( P < 0.05 \) vs. room air. †\( P < 0.05 \) vs. NT.

DISCUSSION

The present study demonstrates that the depressed ventilatory response to hypoxia observed in HT newborn piglets is associated with a significant reduction in the release of extracellular levels of Glu in the NTS.

During normoxia a change in the breathing pattern characterized by a significant decrease in burst frequency and a trend to an increase in the area activity (area under the curve) of the phrenic nerve was observed in the HT newborn piglets. Similar findings were reported in anesthetized, paralyzed, and mechanically ventilated cats while brain temperature decreased from 37.5 to 30.5°C (17). Furthermore, a significant decrease in respiratory rate was also described in anesthetized rats when their body temperature decreased from 37.5 to 35°C (7).

The initial hyperventilation observed during hypoxia is attributed to peripheral chemoreceptor stimulation and is influenced by change in body temperature, which has a marked effect on carotid nerve afferent discharges. An increase in temperature augments the frequency of carotid nerve afferent discharge, whereas cooling reduces it (24). In the present study the initial increase in minute ventilation with hypoxia was eliminated in HT newborn piglets. This is in agreement with...
the findings of Ceruti (3), who reported that the increase in ventilation at 1 min of hypoxia was also abolished in newborn infants kept in a cool environment.

Afferent input from peripheral chemoreceptors modulates the central release of Glu during hypoxia, which then increases the activity of brain stem respiratory motoneurons in NT adult dogs and cats (12, 23). The initial response and the subsequent hyperventilatory response to hypoxia observed in adult dogs were abolished by carotid body denervation, and this was accompanied by a decrease in the central nervous system (CNS) Glu turnover (12). In sham-operated adult rats, Glu concentration in the NTS increased during hypoxia and gradually returned to the baseline values after the hypoxia was discontinued (25). In contrast, NTS Glu levels did not change with hypoxia in chemodenervated adult rats (25). In addition, in chemodenervated adult dogs in which the initial hypoxic hyperventilation was abolished, the Glu turnover was reduced in the whole brain during normoxia and the increase in the Glu content of the medulla during hypoxia was also eliminated, suggesting that hypoxia modified the medullary content of Glu by afferent stimuli arising from peripheral chemoreceptors (12, 16). The initial increase in ventilation with hypoxia was eliminated in HT newborn piglets, and this was followed by a decrease in ventilation at 15 min of hypoxia. This response is similar to that observed in chemodenervated animals. Therefore, the lack of increase in the NTS Glu levels during HT may be due to decreased peripheral chemoreceptor activity.

In unanesthetized piglets the ventilatory response to hypoxia is markedly depressed after NMDA blockade (21). This suggests that the endogenous excitatory amino acid Glu influences the ventilatory response to hypoxia through NMDA receptors. The initial increase in the phrenic nerve output observed during hypoxia was eliminated by the application of the NMDA blocker to the ventral medullary surface of the brain stem of adult rats (32). Lin et al. (21) demonstrated that the decrease in the ventilatory response to hypoxia after NMDA receptor blockade in piglets occurred during the 1st min as well as after 10 min of exposure to low O₂ concentration. This suggests that Glu may be an important mediator of the initial and late hypoxic ventilatory responses. However, we cannot rule out the possibility that the late hypoxic ventilatory depression is due to a reduction in the release of central excitatory amino acid neurotransmitters, which results in enhancement of the effects of inhibitory neurotransmitters such as GABA. This is supported by the fact that an increase in CNS GABA concentration with hypoxia was observed in chemodenervated adult dogs while the medullary Glu content remained unchanged (16). Preliminary data from our laboratory showed a greater increase in the ventilatory response to hypoxia in HT newborn piglets than in NT animals after intracisternal administration of bicuculline methiodide, a GABA_A receptor blocker (37). This suggests that the decrease in the ventilatory response to hypoxia observed during HT is also modulated by an increased CNS GABA concentration.

The mechanism by which HT modifies the CNS Glu levels has not been completely elucidated. The reduction in CNS extracellular concentration of Glu during hypoxia and HT conditions may be explained by an increase in the reuptake of Glu and/or a decrease in the rate of Glu release from the presynaptic cells (14, 28). In the normal brain, Glu is released by Ca²⁺- and ATP-dependent mechanisms through a conventional vesicular release mediated by NMDA receptor channels (35). It has been proposed that during brain ischemia Glu is released by a non-Ca²⁺-dependent mechanism, which is determined by the change in ionic (Na⁺ and K⁺) gradients during the anoxic depolarization, leading to a release of Glu by reversed operation of the Glu uptake carrier (35).
Although extensive studies of the effect of selectively induced brain cooling on the CNS Glu concentration during brain ischemia are available (2, 10), there are no published data on the role of peripheral chemoreceptor activity in the CNS Glu production during HT. In the present study it is impossible to determine whether the decrease in the NTS Glu concentration observed during hypoxia in HT conditions was modulated centrally or peripherally, since the brain HT was achieved by lowering the whole body temperature.

Because ventilation is tightly linked to metabolism, it is reasonable to expect that the decrease in VO2 during hypoxia in HT may be one of the mechanisms for the hypoxic ventilatory depression observed in HT newborn piglets. However, the fact that the decrease in VO2 with hypoxia was not different between NT and HT rules out this possibility.

During HT an increase in lactic acid production associated with a decrease in pH and base excess has been described (20). In the present study, pH and base excess were not different between groups during normoxia and hypoxia; thus changes in the acid-base status are not responsible for the depressed ventilatory response to hypoxia in HT animals.

The lack of ventilatory response to hypoxia observed in HT newborn piglets could be the result of a generalized depression in synaptic transmission due to brain HT. On the basis of this assumption, it is expected that the ventilatory response to hypercapnia is also depressed during HT. An attenuation in the ventilatory response to hypercapnia has been demonstrated during deep HT in anesthetized animals (22, 29). However, the slope of the CO2 response curve observed during moderate HT did not differ significantly from the NT conditions (4, 15, 30).

The effects of Glu on the central control of the cardiovascular function have been extensively investigated (5, 8, 9, 11). An increase in ABP but not HR was observed after ventriculocisternal perfusion with Glu in adult dogs (5). However, in newborn piglets a significant decrease in HR and unchanged ABP were noted during hypoxia after Glu antagonist administration (21). The effects of Glu agonists and antagonists on the cardiovascular system are controversial in the literature, and this may represent differences in the methodology, drug dosage, and animal models used. In the present study, HT caused a decrease in HR but no change in ABP compared with NT conditions. This is in agreement with a significant decrease in HR and cardiac output and no significant changes in ABP and pulmonary and systemic vascular resistance observed in normoxic young pigs when their core temperature was decreased to 31.5°C (36). The fact that changes in ABP and HR with hypoxia were not different between NT and HT groups makes it unlikely that the hypoxic ventilatory depression observed in HT animals was due to cardiovascular changes.

In conclusion, the results of this study suggest that the marked hypoxic ventilatory depression observed in HT piglets is in part due to the lack of increase in CNS Glu concentration.

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