Curtained respiration by repeated vs. isolated hypoxia in maturing piglets is unrelated to NTS ME or SP levels

KAREN A. WATERS, ANDRé LAFERRIÈRE, JULIE PAQUETTE, CYNTHIA GOODYER, AND IMMANUELA R. MOSS Developmental Respiratory and Endocrinology Laboratories, Department of Pediatrics, McGill University, and The Montreal Children's Hospital Research Institute, Montreal, Quebec, Canada H3H 1P3

Waters, Karen A., André Laferrière, Julie Paquette, Cynthia Goodyer, and Immanuela R. Moss. Curtained respiration by repeated vs. isolated hypoxia in maturing piglets is unrelated to NTS ME or SP levels. J. Appl. Physiol. 83(2): 522–529, 1997.—In early development, respiratory disorders can produce recurring hypoxic episodes during sleep. To examine possible effects of daily repeated vs. isolated hypoxic hypoxia, cardiorespiratory functions and central, respiratory-related neuromodulator levels in 21- to 32-day-old, chronically instrumented, unsedated piglets were compared between a fifth sequential daily hypoxia and an isolated hypoxia (10% O2–90% N2 for 30 min). Diaphragmatic electromyographic activity, heart rate and arterial pressure, and pH and gas tensions were measured. In vivo microdialysis, via chronically implanted guides, served to sample interstitial substance P (SP) and methionine-enkephalin (ME) at the level of the respiratory-related nucleus tractus solitarii (NTS). Compared with an isolated hypoxia, repeated hypoxia resulted in 1) lower respiratory frequency (f), ventilation equivalent, and arterial pH, higher arterial Po2 during hypoxia, and lower f in recovery from hypoxia; and 2) increased SP concentrations but no change in ME concentrations. We conclude that, in these maturing swine, repeated vs. isolated hypoxic exposure curtails respiratory responses to hypoxia by a mechanism(s) unrelated to SP or ME levels at the NTS.

repeated hypoxic exposure has been reported to produce physiological responses that differ from those to an isolated hypoxic challenge. For example, severe hypoxia, imposed repeatedly every few minutes, suppresses arousal in lambs (4) and reduces respiratory responses in piglets (22). The mechanisms underlying the altered responses to this type of repeated hypoxia are obscure. Because respiratory disorders in infants can produce recurrent daily episodes of hypoxia, it is important to establish whether such a repeated-hypoxic pattern also alters cardiorespiratory responses. In the present study, we have set out to answer this question by using a chronically instrumented, unsedated piglet model (20).

Because hypoxia has been documented to increase extraneuronal levels of neuromodulators (11, 12, 21, 24) that are related to cardiorespiratory control (1, 13, 17, 23), changes in these neurochemical systems could underlie the altered responsiveness to repeated vs. isolated hypoxia. In vivo microdialysis provides a means to measure extraneuronal concentrations of such neurochemicals in correlation with physiological responses to an imposed stimulus. Among the central respiratory regions that regulate respiration, the respiratory-related nucleus of the solitary tract (NTS) is an important relay station that plays an integrative role in cardiorespiratory control (2) and is amenable to microdialysis, thus far under anesthesia (11, 12, 21, 24).

Substance P (SP) and methionine-enkephalin (ME) are neuromodulators that, respectively, stimulate and suppress respiration (15). These neuromodulators are colocalized within the central nervous system (18) and exist abundantly within the NTS (7, 25). Extraneuronal SP and ME levels at the NTS of anesthetized adult rabbits (21) and maturing piglets (24) have been shown to increase during acute hypoxia. Changes in SP and ME levels, or in their relative dominance at the NTS or at other respiratory-related brain regions, could contribute to an altered respiratory response to repeated vs. isolated hypoxia.

Thus the hypothesis tested in the present study is that daily bouts of hypoxic hypoxia (henceforth termed “hypoxia”) diminish the ventilatory responsiveness to a subsequent hypoxia and that a relative overabundance of inhibitory vs. excitatory neuromodulatory influence at the NTS contributes to this suppression. To test this hypothesis, the present study assessed cardiorespiratory responses to repeated vs. isolated hypoxia concurrently with interstitial SP and ME concentrations at the NTS in chronically instrumented, unsedated, 21- to 32-day-old piglets. Maturing piglets were selected because, relative to younger piglets, they display a more excitatory respiratory response to acute hypoxia (17) and a declining opioid content at the NTS (25) that, in other species, is accompanied temporally by a maturing SP system (15). Thus, if our hypothesis were correct, the age range selected for study was more likely than younger ages to exhibit a clear effect of repeated hypoxia on the respiratory response and neuromodulatory balance, as expressed by a reversal from its predominantly excitatory state to a predominantly inhibitory state.

METHODS

Animals and Instrumentation

Sixteen Yucatan Miniature Swine (Charles River) from 6 litters, 4 females and 12 males, were studied between 21 and 32 postnatal days. The study was approved by the McGill Facility Animal Care Committee and complied with the Canadian Guide for the Care and Use of Experimental Animals and the National Research Council Guide for the...

Surgery was undertaken 2 days before the first experiment. The piglets were anesthetized with 1–2% isoflurane in a 1:1 mixture of N2O–O2 and remained intubated and anesthetized throughout surgery. A right femoral arterial cannula served to measure arterial pressure (Gould Electronics, Cleveland, OH), pH, and gas tensions (at body temperature; Radiometer ABL3, Copenhagen, Denmark). The cannula, tunneled subcutaneously and exteriorized at the flank, was protected within the pocket of a custom-made jacket that the piglets wore after surgery. Transdiaphragmatic (EMGdi) and submental electromyographic (EMGsm) electrodes were inserted. The piglets were then positioned in a stereotaxic frame with the incision bar set 20.0 mm below the interaural plane (David Kopf Instruments, Tujunga, CA). For the implantation of electrocorticographic (ECoG) electrodes and a chronic microdialysis guide cannula, a portion of the cranium was exposed and stripped of periosteum. Five stainless steel screws were inserted through the skull to abut against the cortex. The bare end of a Teflon-coated stainless steel wire (Cooner Wire, Chatsworth, CA) was wrapped around each cranial screw and connected to a plastic electrical socket (Kent Scientific, Litchfield, CT). To record the electrocorticogram (EOG), knotted wires were inserted into the muscle at the outer canthus of each eye. The electromyographic (EMG) and EOG electrodes were tunneled to the back of the head and connected to a second plastic socket. A 1-mm hole was drilled through the skull 5 mm posterior and 1.5–2.4 mm lateral to the brema, and a microdialysis guide cannula (CMA 10, Carnegie Medicin, Stockholm, Sweden) was inserted at a 22° angle to a depth 3 mm short of the target for the microdialysis probe at the NTS. Orthodontic resin served to fix the guide cannula, to cover the cranial screws, and to attach the pedestal sockets to the skull.

Each piglet received iron dextran complex (100 mg im) on the day of surgery as well as intravenous cephalizin and oral/rectal acetaminophen on the day of surgery and for 2 days postoperatively. Forty-eight hours after surgery and throughout the remainder of the study, the piglets exhibited normal feeding and playful behavior as well as a daily weight gain of 61.3 ± 16.9 (SE) g.

Experimental Environment and Measurements

For each study, the piglet was placed in a sling within a sealed box constructed of 1-cm-thick Perspex. The pedestal sockets were connected via a plug fixed within a wall of the box to a polygraph (Gould Electronics) for band-pass filtering (ECoG, 1–300 Hz; EMG, 0.1–3 kHz) and amplification. The EMGdi signal was full-wave rectified and integrated (Bioinstrumentation Services, Univ. of Texas Southwestern Med. Center, Dallas, TX). The box and piglet's rectal temperatures were monitored throughout (TH-5, sensitivity 0.1°C, Physi-techn Instruments, Clifton, NJ). Box temperature was maintained at 30.4 ± 0.3°C (the thermoneutral temperature of piglets of this age) by adjusting the temperature of the heated gases in the box. Box O2 and CO2 were sampled at a rate of 100 ml/min, desiccated with CaSO4 (Drierite), and analyzed continuously (Ametek, Pittsburgh, PA); fractional CO2 (Fco2) in the box was kept at ≈0.2% throughout. All signals were recorded on paper, taped (Hewlett-Packard, Rockville, MD), and digitized [model RSC-16 analog-to-digital board, RC Electronics, Santa Barbara, CA; and custom-designed software, with sampling rates of 256 Hz for ECoG and raw EMGdi signals and 64 Hz for EOG, arterial pressure, and integrated EMGdi signals (16)].

For each microdialysis experiment, the guide obturator was removed, and a microdialysis probe (CMA 10, shaft length 70 mm, external diameter 0.7 mm, membrane length 3 mm) was inserted through and 3 mm beyond the guide to a depth of 44.3–46.3 mm from the dural surface and left in place for the duration of that experiment. The probe was perfused at 2 µl/min with artificial cerebrospinal fluid containing (in mM) 133 NaCl, 3.0 KCl, 2.0 CaCl2, 0.7 MgCl2, 2.4 NaHCO3, and 3.7 glucose, pH 7.36, with 0.2% bovine serum albumin to prevent adherence of peptides to the probe membrane and 0.03% bacitracin to reduce peptidase activity. Sixty-microliter samples were collected at 4°C for 30 min each (CMA 170 fraction collector). The time lag between the dialysis and its collection (calculated from the dead space of the collecting system at the flow rate used) was 10 min; this time lag was taken into account throughout the experimental protocol so as to match the respiratory data collection. The in vitro recovery of SP and ME, by using this probe and flow rate, was 2.4 ± 0.2 and 6.1 ± 0.3%, respectively. These values are within the range reported by others (21, 24).

Hypoxic Experimental Protocol (Fig. 1)

After a 90-min stabilization period for animals in the box, baseline measurements in normoxia were collected. These included a 30-min microdialysate sample, all physiological functions for a 10-min period, and an arterial sample for pH and gas tension measurements. The piglet was then exposed to 10% O2–90% N2 for 30 min, during which all physiological functions were collected, arterial pH and gas tensions were measured, and a microdialysate sample was obtained. A 1-h recovery period ensued, during which two 30-min microdialysates were collected, each accompanied by an arterial pH and gas tension analysis as well as by an acquisition of all physiological functions, for 10 min in early recovery and for 5 min in late recovery. At the end of the experiment, the microdialysates were frozen and stored at −70°C for subsequent analysis.

Study Groups

Each piglet underwent five consecutive daily sessions in the box, at the same time each day. For the repeated-hypoxia
group, each daily session included the 30-min exposure to hypoxia, of which the first and fifth daily sessions were recorded. The isolated-hypoxia group underwent sessions with hypoxia on days 1 and 5 only, both of which sessions were recorded. On days 2–4, the isolated-hypoxia group underwent similar experimental sessions but without exposure to hypoxia. In both groups, microdialysis was performed twice, on days 1 and 5, after acute probe insertion. Such repeated insertion, in contrast to chronic probe implantation, has been reported to result in less tissue damage at the probe site and in more reproducible neurochemical concentrations over time (5, 6). After the last experimental day, each piglet was euthanized with an overdose of pentobarbital sodium and perfused with 10% buffered formaldehyde. Each piglet underwent necropsy to confirm health and proper lead placements. Each brain was removed, sectioned (40 µm) in a cryostat (IEC Minotome, Needham Heights, MA) and stained with cresyl violet for definition of the precise site at which the microdialysis had been carried out.

Data Processing and Quantification

The integrated EMGdi signal was analyzed by using the standard method of this laboratory (16). Briefly, the digitized recordings were replayed, and segments including 5–10 consecutive breaths were analyzed for each 2-min record. A window defining the start and end of each sampled breath was drawn on a scrolled, integrated-signal display, from which the following functions were measured and averaged for each group of breaths: the maximal amplitude of the envelope (peak EMGdi amplitude), the slope of the integrated EMG signal from its onset to 100 ms (EMGdi slope), the total area under the integrated EMG signal (EMGdi area), the interval between successive breaths (TT) and its inverse, respiratory frequency (f), ventilation equivalent (Ve; EMGdi area × f), the total duration of the EMG envelope (EMGdi duration), and the interval between the end of one EMG burst and the onset of the next burst (TT − EMGdi duration). Mean values for fractional O2 (F O2), heart rate, and arterial pressure were also furnished by the analysis software for the same time segments. Because, both behaviorally and electrographically (ECO2, EOG, EMGsm), the piglets were awake during the hypoxic exposures and the recoveries therefrom, no further sleep-wake analyses were performed.

Peptide Separation by High-Pressure Liquid Chromatography (HPLC)

The thawed microdialysates underwent peptide separation by HPLC ( Dionex; 30- min runs by use of a Zorbax RP 300-C18 reverse-phase column at 34°C and an acetonitrile-to-H2O progressive gradient of 15–26.25% plus 0.1% trifluoroacetic acid). The temporal elutions of SP and ME were deduced from concentrated peptide standards (2.5 µg/ml each) that were visible at 215-nm ultraviolet wavelength. To allow for variability in elution among samples, the desired fractions were collected over ≈4-min periods, around the predetermined 5.0 ± 0.5 and 20.0 ± 1.0 (SD)-min elution times for ME and SP, respectively. The fractions were refrigerated overnight at 4°C, lyophilized the following day (SC110, Savant Instruments, Farmingdale, NY), and stored until radioimmunoassay (RIA) at −70°C. The percent peptide recovery from these procedures was 73 ± 10.8% for SP and 83 ± 9.2% for ME.

RIAs

All samples from each piglet were analyzed together. RIAs for SP and ME were performed by using kits (Incstar, Stillwater, MN). Briefly, the lyophilized samples were reconstructed in RIA buffer to 420 µl and then assayed in duplicate. Because ME levels in each 60-µl microdialysate were at the low limit of the RIA sensitivity, each two consecutive samples (except the 7th) were combined (Fig. 1), and 8 pg of standard ME were added to each paired sample to ensure that the measured concentration would fall within the sensitive portion of the standard curve. The samples containing SP did not require any combination or special treatment. Samples with unknown peptide concentrations, or with known standard peptide concentrations, were then mixed with 100 µl of anti-peptide antibody and with 100 µl of 125I-labeled peptide in borosilicate glass tubes. The tubes were incubated overnight at 4°C. The following day, 100 µl of γ-globulin and 500 µl of saturated ammonium sulfate were added to each tube, and the tubes were incubated at room temperature for 15–25 min. All tubes from the same assay were centrifuged together, after which the supernatant was aspirated and the radioactivity in the residual pellet was counted (Packard auto-gamma counter). The unknown peptide levels in the samples were calculated from the standard curves by using the software supplied with the counter. The sensitivity limits of the assays averaged 10.1 pg/ml (2 pg/tube) for SP and 4.35 pg/ml (1.1 pg/tube) for ME. The intra- and interassay variabilities, respectively, were 6.7 and 9.6% for SP and 5.5 and 8.1% for ME. The manufacturer-quoted antibody cross-reactivities for SP were <0.002% with ME, leucine-enkephalin, β-endorphin, endo- and physalaemin; for ME these were 2.8% with leucine-enkephalin, 0.1% with α-endorphin, and <0.002% with SP, β-endorphin, porcine dynorphin-(1–13) and α-endorphin.

Statistical Analyses

Physiological measurements. For each recorded session, the baseline respiratory and cardiovascular data were averaged over the baseline normoxic period, for each consecutive 6-min interval during the 30-min hypoxic period, and for the early and late recovery periods (Fig. 1). Results during hypoxia and recovery were normalized to baseline and analyzed by a two-way analysis of variance with one repeated measure to determine the effects of time (in hypoxia or recovery) and of treatment (repeated vs. isolated hypoxia) on each physiological function. Arterial pH and gas tensions and rectal temperature underwent similar analyses, albeit by using raw rather than normalized data. When significant treatment-related main differences or interactions were detected for any variable, pairwise comparisons of group means at each hypoxic or recovery interval were made by Scheffe’s test.

The same analysis of the data collected from each study group during the initial hypoxic exposure and recovery did not reveal any differences in respiratory or cardiovascular functions, arterial pH or gas tensions, or rectal temperatures between the groups. Thus the piglets entering the repeated- or the isolated-hypoxia protocol were the same.

Neuromodulator measurements. The analysis of SP and ME levels (the latter corrected for the standard added for RIA) simulated that of the physiological functions, in that, for each microdialysis experiment, SP and ME concentrations during hypoxia and recovery were normalized to baseline level and then subjected to a two-way analysis of variance with one repeated measure (time in hypoxia and recovery: repeated vs. isolated hypoxia) by using a least squares means procedure to account for missing observations. When treatment-related effects were found, comparisons between the two treatment groups were made at hypoxia and recovery by using paired t-tests. Similar to the physiological results, no differences were found between the piglets assigned to each of the study groups in either SP or ME concentrations during
the initial hypoxic exposure and the recovery therefrom, indicating that all piglets were the same on entering the study protocols.

All analyses were performed by using a computer software package (SAS Institute, Cary, NC). Unless otherwise stated, all results are presented as means ± SE.

RESULTS

The focus of this section is on a comparison of responses to hypoxic exposure on study day 5 in the piglet group that had experienced hypoxia repeatedly with those in the group that did not.

Piglet Characteristics

There was no difference between the repeated- and isolated-hypoxia piglet groups on study day 5 at baseline normoxia in age, sex distribution, body weight, daily weight gain, hemoglobin concentration, rectal temperature, f, or heart rate or in arterial pressure, pH, and gas tensions (Table 1).

Effects of Repeated Vs. Isolated Hypoxia on Respiratory and Cardiovascular Functions (Figs. 2–4)

During the hypoxic period, all piglets exhibited biphasic responses in peak EMGd1 amplitude and Veq, as characterized by a rapid initial increase followed by a decrease over the remaining time in hypoxia [time effect: F(4,44) = 5.62, P = 0.001; F(4,44) = 3.32, P = 0.021, respectively]. During the early and late recovery periods, there were no differences between the study groups in either peak EMGd1 amplitude or Veq. Repeated vs. isolated hypoxia diminished the Veq response throughout the hypoxic period [main effect of treatment during hypoxia: F(1,11) = 9.37, P = 0.007]. In contrast, f was altered differentially by repeated vs. isolated hypoxia [interaction between time and treatment in hypoxia: F(4,44) = 5.33, P = 0.001]. During the initial 6-min hypoxic interval, f rose to the same extent in repeated and isolated hypoxia, but, thereafter, it increased proportionally less in the repeated-hypoxia group (post hoc pairwise comparisons, P < 0.05); f also remained relatively lower in the repeated-hypoxia group during the recovery periods [main effect of treatment in recovery: F(1,11) = 4.84, P = 0.05]. The reduced f response was mirrored by EMGd1 duration [interaction of treatment and time in hypoxia: F(4,44) = 4.32, P = 0.005], which decreased to the same extent in early repeated and isolated hypoxia but to a lesser extent in late repeated vs. isolated hypoxia (post hoc pairwise comparisons, P < 0.05) as well as throughout recovery [main effect of treatment during recovery: F(1,11) = 6.19, P = 0.026]. In addition, Tr – EMGd1 duration decreased to a lesser extent in the repeated- vs. isolated-hypoxia group throughout the hypoxic challenge [main effect of treatment during hypoxia: F(1,11) = 8.30, P = 0.008] but insignificantly so in recovery (Fig. 2).

The attenuated respiratory responses to repeated vs. isolated hypoxia were accompanied by a lesser increase in arterial pH and a lesser decrease in arterial Pco2 during repeated hypoxia [interaction between time and treatment: F(1,12) = 6.05, P = 0.030; F(1,12) = 4.18, P = 0.061, respectively]. Neither rectal temperature (unchanged during hypoxia) nor arterial Po2, however, differed between the repeated- and isolated-hypoxia groups at any time (Fig. 3). Heart rate and arterial pressure were unaffected by repeated vs. isolated hypoxia (Fig. 4).

Interstitial NTS Levels of SP and ME with Repeated vs. Isolated Hypoxia (Figs. 5 and 6)

Of the 16 microdialysis probe placements, the dialysis membrane of 14 probes either intersected or came within 300 µm of the NTS (Fig. 5). The SP and ME results from the two failed placements (1 in each study group) were excluded from analysis.

SP concentrations were stable over the 1-h baseline period in normoxia and did not differ between the study groups. SP normalized to baseline (Fig. 6) displayed relatively increased levels in the repeated- vs. isolated-hypoxia group [main treatment effect: F(1,12) = 4.75, P = 0.052], and group differences were significant during the two recovery periods (P = 0.031 and 0.020, respectively). SP and ME levels remained unchanged over time. In addition, ME levels were not different in the repeated- vs. isolated-hypoxia group (Fig. 6).

DISCUSSION

This study combined a detailed assessment of respiratory and cardiovascular functions with measurements of interstitial SP and ME concentrations at the level of the NTS by repeated in vivo microdialysis in chronically instrumented, unseeded, maturing piglets (20). The study shows that repeated daily exposure to hypoxia results in an attenuated respiratory response to a subsequent acute hypoxic insult. Furthermore, such repeated vs. isolated hypoxia also increases the interstitial concentrations of SP at the NTS.

The repeated- and isolated-hypoxia groups underwent identical experimental protocols over the 5 consecutive study days, except for a hypoxic resired-gas exposure, respectively, on study day 5 at baseline normoxia.

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**Table 1. Characteristics and physiological functions in piglets in repeated-hypoxia and isolated-hypoxia groups on study day 5 at baseline normoxia**

<table>
<thead>
<tr>
<th></th>
<th>Isolated-Hypoxia Group (n = 8)</th>
<th>Repeated-Hypoxia Group (n = 8)</th>
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<tbody>
<tr>
<td>Age, days</td>
<td>30 ± 1</td>
<td>30 ± 1</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>6:2</td>
<td>6:2</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>3.6 ± 0.3</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>Daily weight gain, g</td>
<td>47 ± 26</td>
<td>76 ± 22</td>
</tr>
<tr>
<td>Hb, g/dl</td>
<td>10.3 ± 0.6</td>
<td>10.4 ± 0.5</td>
</tr>
<tr>
<td>Rectal temperature, °C</td>
<td>38.6 ± 0.4</td>
<td>38.8 ± 0.2</td>
</tr>
<tr>
<td>f, breaths/min</td>
<td>36 ± 6</td>
<td>40 ± 7</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>148 ± 23</td>
<td>152 ± 13</td>
</tr>
<tr>
<td>Arterial pressure, mmHg</td>
<td>83 ± 10</td>
<td>94 ± 4</td>
</tr>
<tr>
<td>pHa</td>
<td>7.38 ± 0.01</td>
<td>7.37 ± 0.01</td>
</tr>
<tr>
<td>PaO2, Torr</td>
<td>96.2 ± 9.8</td>
<td>95.9 ± 7.1</td>
</tr>
<tr>
<td>PaCO2, Torr</td>
<td>42.2 ± 1.7</td>
<td>39.7 ± 1.6</td>
</tr>
</tbody>
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Values are means ± SE. n, No. of animals; M, male; F, female; Hb, hemoglobin; f, respiratory frequency; pHa, arterial pH; PaO2 and PaCO2, arterial Po2 and Pco2, respectively.
days 2–4. Furthermore, all piglets had the same characteristics and the same cardiorespiratory and neuro-modulatory responses to the initial hypoxia as well as at baseline normoxia on study day 5. Therefore, there can be no doubt that any difference between the groups during and after the hypoxia on study day 5 was due to the repeated vs. the isolated nature of the hypoxic exposure.

The attenuated respiratory responses to the repeated vs. the isolated hypoxia were substantial. Although assessed by EMG and not by airflow measurements, this attenuation revealed both an altered breathing pattern and relative hypoventilation. The latter was evidenced by the relatively lower arterial pH and higher arterial PCO2 in the repeated-hypoxia group.

The present study is different from previous ones (4, 22) in the severity and modality of the repeated hypoxic stimulus. For example, unsedated, tracheotomized lambs that were exposed to severe hypoxia (FO2 = 0.05) repeatedly during each of 100 successive quiet and active sleep epochs displayed a delay in arousal with repeated hypoxia, indicating tolerance to the hypoxic stimulus; at the same time, the EMGdi and f measurements increased with the repeated hypoxia, a discrepant finding to ours that is difficult to explain (4). In a previous study from our laboratory in unsedated piglets (22), repeated hypoxia, compared with a continuous hypoxic stimulus, produced a relative respiratory attenuation, but the hypoxic stimulus was more severe than the present one (6% instead of 10% O2 in N2) and was repeated seven times over a 30-min period in alternation with brief exposures to normoxic mixtures. Thus the unique finding in the present study is that even a relatively moderate hypoxic stimulus, imposed for a short fraction of each successive 24-h period, could elicit a relative hypoventilation compared with that incurred by a similar, but isolated, hypoxia.

Of the mechanisms that could contribute to the attenuated respiratory responses to repeated daily hypoxia, this study focused on the possible central role of SP and ME. Whereas the neurotachykinin-1-SP system has been shown in several species to undergo rapid proliferation during development, and to be abundant in the NTS and other respiratory-related brain stem regions throughout life (reviewed in Ref. 15), its development and distribution in the pig brain are unknown. With regard to the excitatory influence of SP on respiration, preliminary information in swine is
available: intracerebroventricular injection of SP in unsedated, chronically instrumented piglets of the same age range as that used in the present study increases peak EMGdi amplitude in normoxia (A. Laferrière, D. Fung, K. A. Waters, and I. R. Moss, unpublished observations). This finding confirms the reported excitatory effect of SP superfused on the dorsal medullary surface of anesthetized or decerebrate rabbit pups (23) or microiontophoresed and microinjected, respectively, into the NTS of adult, anesthetized cats (13) and rats (1).

Such stimulatory effects could be mediated by increased extraneuronal levels of SP during hypoxia, perhaps by its enhanced release from neurons. Indeed, in the present study, a daily repeated exposure to relatively modest hypoxia increased these concentrations in the NTS throughout the hypoxic and subsequent recovery. These findings are in general agreement with previous reports using repeated hypoxia and in vivo microdialysis in cats (11) and rabbits (21). However, whereas in our study the magnitude of this increase was modest and extended to the posthypoxic period, the previously reported increases in SP were substantial and restricted to the hypoxic period (11, 21). This discrepancy can perhaps be explained by differences in the modality and severity of the repeated hypoxic stimulus as well as in the developmental stage, arousal level, and species of the subjects.

In our study employed repeated daily exposures to 0.10 FO₂ for 30 min in maturing, awake piglets, whereas the previous studies employed two to three exposures over 10 or 30 min to 0.09 FO₂ in adult, pentobarbital sodium-anesthetized, paralyzed, and artificially ventilated cats and rabbits (11, 21). It is thus possible that SP levels exhibited a lesser and more temporally extended rise because of the more moderate stimulus, the longer intervals between exposures, and the more physiological state of our experimental subjects. It is also possible that the SP system had not attained its full development in our maturing (but not adult) piglets.

Because the increased interstitial NTS levels of SP would be expected to have a stimulatory effect on respiration, the attenuated respiratory responses seen with the repeated hypoxic stimulus cannot be explained by this increase. On the other hand, a reduced density and/or affinity of SP receptors in the NTS might explain the apparent ventilatory insensitivity of these piglets to the increased interstitial levels of SP with repeated hypoxia. Therefore, the effect of repeated

Fig. 4. Heart rate and arterial blood pressure responses to repeated vs. isolated hypoxic challenge in chronically instrumented, unsedated piglets on study day 5. All functions are normalized to baseline values (dotted line). See text for statistical analyses.

Fig. 5. Schematic representation of microdialysis probe placements on sagittal sections of piglet medulla. Nos. to right of each section, distance from midline. Probe placements are drawn to scale. Open symbols, isolated-hypoxia group; solid symbols, repeated-hypoxia group; hatched areas, NTS; V, 4th ventricle.
hypoxia on the regulation of neurotachykinin-1 receptors merits exploration.

The reduced respiratory responses seen with repeated hypoxia cannot be attributed to changes in extraneuronal concentrations of ME because they did not increase significantly in response to either isolated or repeated hypoxia. These results differ from those obtained previously in our laboratory (24), in which the same level and duration of an isolated hypoxic exposure produced a significant increase in interstitial ME-like immunoreactivity at the NTS of piglets of a similar age range. This discrepancy might result from several factors. First, the piglets used by Yan et al. (24) were anesthetized, whereas those used in the present study were conscious. The presence of anesthetics, as already discussed with regard to the SP results, appears to greatly influence the extent of neurochemical release by hypoxia. Second, the ME measured by Yan et al. (24) underwent RIA directly from the microdialysates, that is, without separation by HPLC as performed in the present study. Hence it is likely that, in addition to the pentapeptide itself, other, similar moieties (e.g., longer peptides or breakdown products) were also recognized by the antipeptide antibody used by Yan et al. and were measured together with ME, thus possibly increasing the ligand concentration estimates in that study.

The lack of correlation between the extraneuronal ME levels and the respiratory attenuation with repeated hypoxia may be related to the physiological role of this opioid system at that stage in ontogeny. Both the enhancement of respiratory functions by a specific antagonist to the \( \delta \)-opioid receptor (the receptor for ME) and the baseline ME content at the NTS have been shown to be much lower in animals in this maturing age range than in young neonatal swine (17, 25). Therefore, whereas the \( \delta \)-opioid system in early postnatal life contributes to respiratory suppression even with isolated hypoxia (17), this system becomes less important with age and may play a negligible role in the respiratory response to repeated hypoxia in the maturing age group studied here. The high variability of ME levels in this conscious age group (perhaps because of >3.5-h suspension in the sling) is a factor absent in anesthetized piglets (24).

Aside from the NTS, SP or ME ligand levels and their receptors in other areas could be functionally related to the effects of repeated vs. isolated hypoxia. Additional neuromodulatory systems at respiratory-related regions could also be important in determining the properties of the respiratory oscillator (8) in repeated hypoxia. Such systems might include the ubiquitous \( \gamma \)-aminobutyric acid or adenosine, both shown to suppress breathing (e.g., Refs. 8 and 19).

Of the mechanisms unrelated to neuromodulators that might contribute to the attenuated respiration seen during repeated hypoxia, hypometabolism (14) has been considered. This adaptation, typical of neonates (14), reduces the need for \( O_2 \), thus providing protection against its scarcity during hypoxia. In the present study, this mechanism seems unlikely in view of the unchanged rectal temperature observed in the repeated- vs. the isolated-hypoxia group. The lack of a metabolic response specific to repeated hypoxia appears to be unrelated to the age of the subject or to the nature of the repeated hypoxic stimulus. Younger piglets that do display lowered oxygen consumption and rectal temperature during a 30-min, 10% hypoxic stimulus (22) also diminish respiration during brief, repeti-
tive hypoxic challenges (22) without further reduction in metabolism. Hypoventilation during repeated hypoxia, combined with insufficient hypometabolism, may increase the vulnerability of infants and young children suffering from recurrent rebreathing episodes (9) or upper airway obstruction (3).

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Address for reprint requests: I. R. Moss, Depts. of Pediatrics and Physiology, McGill Univ., The Montreal Children’s Hospital, Suite BB-53, 2300 Tupper St., Montreal, Quebec, Canada H3H 1P3 (E-mail: mdimd@musica.mcgill.ca).

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