HIGHLIGHTED TOPIC | Oxygen Sensing in Health and Disease

Intermittent hypoxia induces transient arousal delay in newborn mice

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Submitted 30 July 2003; accepted in final form 22 October 2003

Intermittent hypoxia induces transient arousal delay in newborn mice. J Appl Physiol 96: 1216–1222, 2004. First published November 14, 2003; 10.1152/japplphysiol.00802.2003.—Previous studies suggested that defective arousal might be a major mechanism in sleep-disordered breathing such as sudden infant death syndrome and obstructive sleep apnea. In this study, we examined the effects of intermittent hypoxia (IH) on the arousal response to hypoxia in 4-day-old mice. We hypothesized that IH would increase arousal latency, as previously reported in other species, and we measured the concomitant changes in ventilation to shed light on the relationship between breathing and arousal. Arousal was scored according to behavioral criteria. Breathing variables were measured noninvasively by use of whole-body flow plethysmography. In the hypoxic group (n = 14), the pups were exposed to 5% O2 in N2 for 3 min and returned to air for 6 min. This test was repeated eight times. The normoxic mice (n = 14) were constantly exposed to normoxia. The hypoxic mice showed a 60% increase in arousal latency (P < 0.0001). Normoxic controls showed virtually no arousals. IH depressed normoxic ventilation below baseline prehypoxic levels, while preserving the ventilatory response to hypoxia. The breathing pattern and arousal responses recovered fully after 2 h of normoxia. We conclude that IH rapidly and reversibly depressed breathing and delayed arousal in newborn mice. Both effects may be due to hypoxia-induced release of inhibitory neurotransmitters acting concomitantly on both functions.

Arousal from sleep is an important protective response that prevents hypoxemia during apneas or impaired gas exchange. Arousal permits the initiation of an appropriate behavioral response, such as head turning, which is particularly important for the resolution of positional asphyxia or obstructive sleep apnea (OSA) secondary to a face-straight-down sleeping position. Previous studies showed that infants who had survived a life-threatening event with OSAs had abnormal arousal responses to hypoxemia (14) and that infants who subsequently died from sudden infant death syndrome (SIDS) showed fewer spontaneous arousals from sleep than did controls (17). These results suggest that defective arousal responses may be major mechanisms in sleep-disordered breathing (SIDS and OSA) in infancy.

A previous experiment in lambs showed that one of the mechanisms that impair the arousal response to hypoxia in newborns is intermittent hypoxia (IH) (15). In these experiments, lambs exposed to IH showed depressed arousal and ventilatory responses to hypoxia during active sleep. Similarly, both ventilatory and arousal responses to hypoxia were blunted in adult dogs with experimentally induced OSA over 3–4 mo (22). Several processes may depress arousability during IH. First, ventilatory efforts (the input from mechanoreceptors) are an important stimulus to arousal from sleep (1, 20, 21) that decreases if ventilation is depressed. Second, depression of the arousal response to IH may be due to habituation. Experiments in adult humans have shown that the arousal respiratory effort threshold during upper airway occlusion is increased by prior sleep apnea (2, 38). Habituation to repeated tactile stimuli has also been shown to depress arousability in infants (29).

The aim of the present experiment was to study the relationship between arousal and ventilatory responses to IH in newborn mice. The rationale for studying mice, rather than other species better described regarding arousal and ventilatory control, is that mice are the preferred mammalian species for genetic studies. Recent results supporting a genetic basis for OSA (34) and SIDS (33, 45) provide a promising avenue for the development of mouse models of sleep-disordered breathing. We hypothesized that IH would increase arousal latency, as previously shown in other species, and we searched for possible relationships between IH-induced changes in breathing and arousal.

METHODS

Animals. Mouse pups were obtained from Swiss female mice (IFFA-CREDO, L’Arbresle, France), housed at 24°C with a 12:12-h light-dark cycle and fed ad libitum. Experimental protocols met the animal research guidelines established by the Institut National de la Santé et de la Recherche Médicale (INSERM; French national institute for health and medical research).

Data acquisition. Breath duration (Ttot, t), tidal volume (VT, μl/g), and ventilation (VE, calculated as VT/Ttot and expressed in μl/s−1·g−1) were measured noninvasively by using whole-body flow barometric plethysmography (6). This plethysmograph was composed of two Plexiglas cylinders serving as the animal (40 ml) and reference (100 ml) chambers, immersed in a thermoregulated water-bath that maintained their temperature at 32.8°C. A 50 ml/min flow of dry air (Bronkhorst Hi-Tec airflow stabilizer, Urlo, Holland) was divided into two 25 ml/min flows through the chambers, thus avoiding CO2 and water accumulation. The differential pressure between the two

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chambers (EFFA transducer, Asnières, France; range ± 0.1 mb) was filtered (bandwidth, 0.05–15 Hz at −3 dB), converted to a digital signal (MacAdios 12-bits converter, GW-Instruments, Somerville, MA) at a sample rate of 100 Hz, and processed by custom-written software (Software Superscope II, GW-Instruments). Calibration was done before each session by injecting 2 µl of air into the animal chamber from a Hamilton syringe. The pressure rise induced by this injection was of similar magnitude to that induced by a newborn mouse.

We recorded body temperature in four additional restrained pups exposed to the same IH protocol. After light anesthesia with isoflurane, a thermocouple probe was placed in the interscapular region (which is the area of highest skin temperature; Ref. 3) through a 2- to 3-mm incision. Previous studies in newborn rats showed that colonic and interscapular temperatures were highly correlated over temperatures ranging from 28 to 40°C (39).

**Arousal from sleep.** Behavior was scored continuously during hypoxia. Arousal was assessed on the basis of previously defined behavioral criteria (4). Behavioral sleep was defined as immobility in the recumbent position (the eyes normally open at 14 days of postnatal age and were therefore closed throughout the study period). Behavioral arousal was defined as a stereotyped motor response characterized by sudden neck and forepaw extension, followed by rising. This coordinated response required cortically mediated activation of the neck, limbs, and thoracic muscles and, therefore, reflected cortical arousal. In contrast, myoclonic twitches characterized by phasic rapid and independent movements of the limbs and tail are components of active sleep and were not considered indicative of arousal (18). Arousal latency was calculated from the transition between air and hypoxic mixture.

In a separate experiment, we assessed arousal using the nuchal electromyogram (EMG). EMG of the nuchal muscles is silent during active sleep, which is the prominent sleep state in newborn mammals (sleep states cannot be determined by electroencephalogram in newborn mice; Refs. 16, 18). A sharp increase in nuchal EMG reflects arousal from active sleep. Behavioral scoring and simultaneous recordings of breathing pattern and EMG were performed in four pups during IH. After light anesthesia with isoflurane, two built-in Kynar hook electrodes (0.24 mm in diameter) were inserted through the skin into the nuchal muscles and secured with adhesive tape. The pup was maintained in contact with an aluminum sheet that served as a ground electrode, inside the plethysmograph. Arousal latency was determined independently 1) during the experiment using the behavioral criteria previously described and 2) after the experiment, on the basis of visual detection of the sharp and sustained increase in nuchal EMG for at least 1 s (30).

**Design.** The experimental design is summarized in Fig. 1. After a waiting time for sleep onset, we recorded baseline VT and VE for 3 min. Then, in the hypoxic group n = 14; postnatal age, 4 days (P4); mean weight, 4.64 ± 0.41 g), the airflow through the plethysmograph was switched to a hypoxic flow (5% O₂–95% N₂) for 5 min and back to air for the next 6 min. This was repeated eight times (total duration, 75 min). After the last hypoxic test, the pups were returned to their litters for 2 h and then tested again. The normoxic control pups (n = 14; age, P4; mean weight, 4.80 ± 0.51 g) were constantly exposed to normoxia while in the plethysmograph; then, to look for possible effects of isolation on breathing pattern, they were subjected to a hypoxic test 2 h after they were returned to their litter. A second control group (n = 6) was exposed to a single hypoxic test after 66 min inside the plethysmograph to control for possible effects of isolation on the arousal response to hypoxia. A third control group (n = 6; age, P5–P6; mean weight, 3.26 ± 0.60 g) was exposed to hypercapnia instead of hypoxia to examine the specificity of hypoxia-induced changes on breathing. Finally, simultaneous measurements of breathing variables and body temperature during IH were done in four pups (age, P5; mean weight, 4.04 ± 0.70 g) and EMG recordings during IH in four pups (age, P8; mean weight, 6.19 ± 1.20 g). Each pup was tested only once.

**Statistics.** All available ventilatory data were used for analyses. The portions of the tracings without individualized breaths or with large drifts exceeding twice the mean amplitude of the volume signal were indicative of movement artifacts and were discarded visually. Ttot, VT, and VE were averaged over successive 30-s periods. The peak VE was determined over the entire hypoxic stimulus. Variables were subjected to ANOVA (Superanova Software, Abacus Concepts, Berkeley, CA), with group (normoxia vs. hypoxia) as the between-subject factor and with test number (1 to 8) and time as within-subject repeated factors. To take into account the heterogeneous correlations among the repeated measurements, we adjusted the degree of freedom using the Greenhouse and Geisser factor (19). We analyzed the effect of the number of tests on arousal and ventilatory responses to hypoxia comparing the values for a given test with the mean value for all subsequent tests (Helmhert contrast). Results are means ± SD in the text and means ± SE in the figures.

**RESULTS**

**Increase in arousal latency during IH.** In the hypoxic group, arousal latency increased during IH (main effect for test: P < 0.0001, Fig. 2). This increase was significant after the first test (contrast test 1 vs. tests 2–8; P < 0.0001) and marginally significant after the second test (P < 0.074), after which arousal latency did not change significantly. Arousal practically never occurred in normoxic controls. After 2 h, the
arousal latencies in the follow-up hypoxic test were not significantly different between the normoxic and hypoxic groups (Fig. 2).

In the second control group, which was exposed to a single hypoxic test after 66 min inside the plethysmograph, the ventilatory and arousal responses were closely similar to the first test in the hypoxic group (all differences nonsignificant, not shown). This showed that isolation in the plethysmograph had no effect on the ventilatory and arousal responses and, therefore, that the increase in arousal latency in the hypoxic group was not caused by isolation.

Behavioral and EMG indexes of arousal. Hypoxia triggered a sharp increase in nuchal EMG, indicating arousal from active sleep (Fig. 3A). The time lag between EMG activation and behavioral arousal was not statistically related to the test number. The mean value was 0.75 ± 1.43 s (range, 1–5 s), which was negligible, compared to the 1-min increase in arousal latency caused by IH. The independent assessments of arousal based on EMG and on behavioral criteria yielded closely similar results (Fig. 3B).

Ventilatory response to repetitive hypoxia. In the hypoxic group, the first hypoxic test was characterized by a biphasic pattern, the initial increase in \( V_E \) being followed by a decline below baseline levels (Fig. 4). This posthypoxic decline in \( V_E \) persisted throughout the 6 min of air breathing, so that \( V_E \) did not return to the initial prehypoxic level. Thus we observed a gradual decrease in \( V_E \) during both the air and the hypoxic periods throughout the IH procedure (with a parallel decrease in \( V_T \), not shown). However, the IH-induced increase in \( V_E \), calculated as the percent increase from the prehypoxic level, increased significantly after the first test and remained constant thereafter (main effect for test: \( P < 0.010 \), contrast test 1 vs. test 2).

Fig. 3. Behavioral and electromyogram (EMG) arousal. A: examples of respiratory and nuchal EMG traces in a newborn mouse. Top signal: behavioral arousal was detected from direct observation. Bottom signal: EMG arousal was determined on the basis of the onset of sustained EMG activity for at least 5 s (in this case, preceded by a small transient EMG increase). Silent EMG interrupted by motor twitches was characteristic of active sleep. A.U., arbitrary units. B: independent assessment of arousal based on behavioral or EMG criteria yielded practically identical results (mean delay < 1 s; see text). Each pup underwent 8 successive hypoxic tests. Each pup is represented by 1 symbol (\( n = 4 \)). Regression line of EMG vs. behavioral arousal: \( y = 0.996x - 0.339 \), \( R^2 = 0.998 \) (indiscernible from the identity line, not represented).

Fig. 4. Ventilatory responses to intermittent hypoxia (5% O\(_2\), shaded areas, \( \bullet \), \( n = 14 \)), compared with normoxic controls (\( \circ \), \( n = 14 \)). Arrows and values indicate mean arousal responses to hypoxia and correspond to arousal latencies shown in Fig. 1. \( V_E \), ventilation. In the first hypoxic test, the initial \( V_E \) increase was followed by a decline below baseline levels and the value did not return to the initial baseline prehypoxic level. After 2 h, \( V_E \) was not significantly different in mice exposed to intermittent hypoxia and in normoxic controls. \( V_T \) returned to the baseline level, and the \( V_T \) response to hypoxia was similar to that obtained during the first hypoxic test. In the normoxic controls, \( V_T \) remained unchanged throughout plethysmographic recording. Values are means ± SE.
tests 2–8, \( P < 0.0001 \), Fig. 5A). The correlations between arousal latency and concomitant \( V_E \), or other breathing variables (\( V_T, T_{tot}, \Delta V_E, \Delta V_T, \Delta T_{tot}, \) where \( \Delta \) denotes change from baseline) were not significant. The time from hypoxia onset to peak \( V_E \) decreased significantly \( (P < 0.008, \text{Fig. 5B}) \), contrary to arousal latency (Fig. 2). Furthermore, the individual correlations between arousal latency and time to peak \( V_E \) were generally not significant, but they were usually negative (one sample \( t \)-test after \( z \)-transform of correlation coefficients, \( P < 0.037 \)).

After 2 h, the hypoxic test yielded similar results in IH-exposed pups and normoxic controls (Fig. 4). Group had no significant effect on \( V_E \), whether as a main effect \( (P = 0.137) \) or in interaction with time \( (F < 1) \). \( V_E \) returned to the baseline hypoxic level, and the hypoxia-induced \( V_E \) peak was similar to that obtained during the first hypoxic test.

Simultaneous measurement of body temperature showed that temperature varied within very narrow limits \( (\sim 0.3°C) \) during IH (Fig. 6). This showed that the reported changes in \( V_E \) during IH were not artifacts caused by temperature changes.

**Ventilatory response to repetitive hypercapnia.** The ventilatory responses to hypercapnia were highly reproducible and did not show any significant changes in normoxic levels or \( V_E \) increases (Fig. 7). This showed that the \( V_E \) changes during IH were specific to hypoxia. During hypercapnia, the stereotyped behavioral response that consistently appeared with hypoxia was not observed, but we cannot rule out that \( CO_2 \) triggered more subtle behavioral changes.

**DISCUSSION**

The main results of this study are that, in newborn mice, IH 1) increased the arousal latency to hypoxia and 2) depressed normoxic ventilation, while preserving the ventilatory response to hypoxia. After 2 h of normoxia, the arousal responses to hypoxia recovered fully and baseline breathing returned to prehypoxic levels.

**Assessment of arousal.** The behavioral and EMG criteria for arousal occurred almost simultaneously. Atony of the nuchal muscles, which is associated with a silent EMG, is characteristic of active sleep. An increase in nuchal EMG activity marked the end of active sleep (which was probably predominant, as this is the case in most mammalian species during the first week of life; Ref. 16). Although our data do not allow a detailed analysis of the arousal sequence (28), they show that the depression in arousal response associated with IH was not
due to inaccurate evaluation of arousal based on behavioral indexes only.

The nearly simultaneous occurrence of EMG and behavioral indexes of arousal found in the present study is congruent with previous data on arousal in human infants subjected to tactile stimuli (28). In infants, the full arousal sequence (syal withdrawal reflex, startle, and full cortical arousal) took place within <0.8 s following the stimulus (28). Nearly complete coincidence between EEG arousal and motor activity detected as tibial anterior EMG activity was also observed in children with sleep apnea syndrome (37). The tiny time lag found in our study between the EMG and behavioral arousals in newborn mice is consistent with these previous studies.

In previous studies in lambs, arousability was assessed by the frequency of arousals during hypoxia, which decreased during active sleep (∼30% of total sleep time) (15). In the present study, sleep states were not identified, but it may be assumed that active sleep was predominant (16). Therefore, the previous study in lambs and the present study in mice, which used different approaches to assess arousal, yield the same conclusion that IH depresses arousability. Moreover, the present study showed that depression of arousal 1) could occur after a single hypoxic episode and 2) recovered fully after a relatively short period.

The increase in arousal latency with IH was probably due to a gradual decrease in the arterial PO\textsubscript{2} threshold at which arousal occurred. Previous studies in 4- to 5-day-old lambs exposed to hypoxia showed that the increase in arousal latency was associated with a decrease in the arterial O\textsubscript{2} saturation value at which arousal occurred (13). However, we were unable to determine the arousal threshold because neither arterial O\textsubscript{2} saturation nor arterial PO\textsubscript{2} can be measured reliably in newborn mice with currently available instruments.

Ventilatory changes during IH. We first addressed the possibility that the decrease in ventilation was caused by measurement drifts. The chamber temperature was kept strictly constant, and additional control experiments in which body temperature was measured continuously during IH showed marginal changes that hardly seem a reasonable explanation to the large concomitant changes in V\textsubscript{E} (see Ref. 6 for the propagation of error in V\textsubscript{T} calculations using plethysmography). Second, we discarded the possibility that repeated stimulation and large periodic increases in V\textsubscript{E} might affect plethysmographic measures, for example through changes in temperature and/or humidity, because pups exposed to intermittent hypercapnia instead of hypoxia did not show the decreasing ventilatory trend obtained with hypoxia. The decrease in normoxic V\textsubscript{E} was thus specific to hypoxia and probably due to the short interstimulus interval, which prevented V\textsubscript{E} from returning to baseline levels between two successive tests.

Animal studies yielded contrasting results on the effects of IH on the V\textsubscript{E} response to hypoxia. This response declined in newborn lambs exposed to IH overnight (15) and in unanesthetized, chronically instrumented piglets (44). In contrast, 2- to 3-day-old rat pups exposed to IH showed no change in the peak V\textsubscript{E} response to hypoxia but exhibited attenuation of the hypoxic ventilatory decline, i.e., IH-exposed pups maintained their V\textsubscript{E} above the normoxic control levels during the hypoxic ventilatory decline (9). Waters and Gozal (42) examined the high sensitivity of IH-induced ventilatory responses to the pattern of stimulus presentation (cycle duration and number). Depending on this pattern, V\textsubscript{E} during the second and subsequent tests may be increased or decreased. Furthermore, whether the hypoxic stimulus is isocapnic or hypocapnic may account for the different effects of IH on the V\textsubscript{E} response to hypoxia, because lowered CO\textsubscript{2} during poikilocapnic IH may depress V\textsubscript{E} after IH (41). These major effects of the IH pattern and of species-related differences hinder comparisons between this and previous studies.

Was the arousal latency increase caused by ventilatory changes? Previous studies in humans indicated that respiratory mechanoreceptor input to arousal centers in the brain make a major contribution to the arousal response to hypoxia (1). Mechanoreceptor input increases with breathing effort, which in our study can be assessed by V\textsubscript{E} or V\textsubscript{T}. Therefore, the decreasing trend in V\textsubscript{E} (and V\textsubscript{T}) values might account for the arousal latency increase. However, this is unlikely given the nonsignificant correlations between arousal latency and V\textsubscript{E} or the other breathing variables. In particular, the time to peak V\textsubscript{E} decreased significantly, contrary to arousal latency. Taken together, the present results suggested that mechanisms other than ventilatory efforts may contribute to arousal and may explain the gradual increase in arousal latency.

The increase in arousal latency with repeated hypoxic stimulation may be regarded as a habituation process (28). Habituation is a learning process whereby repeated presentation of a stimulus lessens the reflex response to this stimulus (12). By definition, habituation is not the result of sensory adaptation but rather represents a learned inhibition of the response to the stimulus. Sensory adaptation at the chemoreceptor level is unlikely because the magnitude of V\textsubscript{E} changes was not diminished in the present study. Habituation is a common process that has been reported, in particular, when using inspiratory resistive loading in dogs (22), lambs (7), and children (26), as well as in infants subjected to tactile (29) or sound stimuli (23). The increase in arousal latency noted in our study fit in well with the observation that habituation to external stimuli depresses arousal, whatever the nature of these stimuli (7, 23, 29).

As with most mammalian species, mouse pups respond to hypoxia by decreasing their metabolism (31). In the present study, this effect probably accounted for the decreased ventilation and the slight decrease in temperature during IH. Therefore, energy depletion at the cellular level may have caused functional impairments in the central structures involved in arousal. Previous studies showed that cortical activity was profoundly altered in 12-day-old piglets exposed to similar levels of IH (43). However, there is no evidence of such changes in newborn mice. In studies of rat hippocampal slices, neuronal activity of the immature brain proved extremely resistant to hypoxia (32). Furthermore, it has not been established that IH affects all arousal-related regions, because vulnerability to hypoxia is highly region-specific (24).

Arousal latency increase and ventilatory decline: a common mechanism? Hypoxia activates several neurotransmitters (or neuromodulators) such as glutamate, which stimulates breathing, and GABA, opiates, and adenosine, which inhibit breathing (36, 40, 42). The platelet-derived growth factor in the nucleus tractus solitarius exerts a strong inhibitory effect on breathing during hypoxia (10, 11). Several of these neurotransmitters are also pivotal to habituation to external stimuli and sleep induction. In particular, adenosine is an endogenous sleep factor that is thought to directly inhibit wakefulness-promoting
neurons, such as cholinergic neurons in the basal forebrain (35). Cortical GABA is involved in habituation of motor responses to new environments (8). Steroid modulation of the GABA_A receptor has been reported to mediate habituation of the evoked midbrain response to repetitive acoustic clicks (5), although this may not occur at early stages of development (25). Taken together, these results suggest that the delayed arousal and decrease in baseline ventilation may be two consequences, relatively independent from each other, of the accumulation of central inhibitory neurotransmitters.

Clinical implications. Arousal impairments induced by IH are potentially hazardous in infant at risks for SIDS, because full behavioral awakening is necessary to initiate withdrawal responses in case of positional asphyxia or apnea. On the other hand, previous studies showed that infants with OSA terminated central and obstructive apneas without arousals (scored according to EEG, submental EMG, and behavioral criteria) (27). This indicates that behavioral arousal is not a necessary condition for OSA termination and that delayed arousal may be a protective mechanism avoiding sleep disruption. Interestingly, after several weeks of nasal continuous positive airway pressure, the proportion of apneas terminated by arousal was larger (27). Our results suggest that abolition of arousal in infants with OSA may be the ultimate stage of a gradual IH-induced increase in arousal latency. Furthermore, the recovery of the arousal response with continuous positive airway pressure in these infants is consistent with the recovery of arousal after 2 h of normoxia in our study. Finally, the fact that infants with OSA had fewer arousals than normal infants during normal breathing (27) cannot be ascribed to depressed responsiveness to apnea-related stimuli but rather to the enduring effects of the hypoxia-induced release of inhibitory neurotransmitters during apneas.

In conclusion, we showed that IH rapidly and reversibly depressed baseline breathing and delayed arousal in newborn mice. These two effects may represent two consequences of hypoxia-induced release of inhibitory neurotransmitters acting concomitantly on breathing and arousal. Arousal to hypoxia is a critical protective mechanism that can be analyzed experimentally in newborn mice and should be considered in mouse models of early impairments in respiratory control (9).

ACKNOWLEDGMENTS

The authors are indebted to Joëlle Adrien (INSERM U288) and Annie Perez (Hôpital Raymond-Poincaré) for helpful advice regarding the EMG studies.

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