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Effect of hypoxia on expiratory muscle activity in fetal sheep

John M. Bissonnette^{1,2}, A. Roger Hohimer¹, and Sharon Knopp¹

¹ Department of Obstetrics and Gynecology, Oregon Health and Science University, Portland, OR 97239, USA

² Department of Cell and Developmental Biology, Oregon Health and Science University, Portland, OR 97239, USA

Abstract

The fetal respiratory response to acute hypoxia is characterized by depression, often to apnea. This study examined the effect of hypoxia on the electromyogram (EMG) of the thyroarytenoid (TA) muscle. Under anesthesia catheters were placed in the fetal sheep carotid artery, fourth cerebral ventricle, trachea and amniotic fluid and wires sewn into the diaphragm and TA muscle. During normoxic episodes of slow fetal breathing (<40 breaths per min) TA EMG activity was phasic beginning immediately after diaphragmatic EMG bursts and ending well before the next burst. This timing is consistent with the post-inspiratory (post-I) phase of the respiratory cycle. Lowering fetal arterial Pa O₂ from ~20mm Hg to ~13 mmHg resulted in arrest of diaphragm EMG and tonic TA activity. Instillation of the (R,S)- -amino-3- hydroxy-5-methylisoxazole-4-propionic acid (AMPA) ionotropic glutamate receptor antagonist 2,3-dihydro-6-nitro-7-sulphamoyl-benzo(f) quinoxaline (NBQX) into the cerebrospinal fluid (CSF) of the fourth ventricle abolished tracheal pressure deflections and diaphragmatic EMG activity. Tonic TA activity, however, could still be evoked by hypoxia. These results indicate that fetal post-I motoneurons are not inhibited by moderate hypoxia and that their tonic activity may be due to a loss of inhibitory input.

Keywords

amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA); Hypoxic ventilatory response; Thyroarytenoid muscle

1. Introduction

Acute hypoxemia in the fetus results in depression of respiratory activity, often to apnea (Boddy et al. 1974); (Maloney et al., 1975). A number of mechanisms for this respiratory depression have been proposed, for reviews see Bissonnette (2000) and Walker et al, (2000). The majority of studies have used tracheal pressure deflections and/or diaphragmatic EMG activity to define fetal respiratory activity. Thus results have been confined to the output from inspiratory neurons. Walker and Harding (Walker et al., 1984) examined the response of the thyroarytenoid muscle (TA) to stimulation of the superior laryngeal nerve during hypoxia. The reflex activity of this laryngeal adductor was not reduced by hypoxia. Their

Corresponding author: John M. Bissonnette, Baird Hall Room 3030C, L-458, Oregon Health and Science University, 3183 SW Sam Jackson Park Rd., Portland OR 97239, USA, bissonne@ohsu.edu.

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study design confined TA EMG recording to the period following nerve stimulation so that continuous activity was not determined. In work primarily aimed at examining the temporal relationship of TA activity to that of the diaphragm, hypoxia did not affect the tonic TA activity seen when the diaphragm was silent (Kianicka et al., 1998). These results suggest that fetal expiratory muscles are not inhibited during hypoxemia. The present studies were designed to determine whether hypoxemia induces tonic expiratory muscle activity. In addition the temporal relationship between the onset of tonic TA activity and the switch from low voltage electrocorticogram (LV-ECOG) to high voltage HCoG (HV-ECOG) which occurs with fetal hypoxemia was examined in order to answer the question: is tonic expiratory muscle activity induced by hypoxemia itself or is it only associated with the HV-ECOG behavioral state? The reciprocal inhibition between inspiratory and expiratory muscle EMGs varies with chemical drive (Sears et al., 1982). In order to determine the effects of CO₂ on expiratory muscle activity the TA EMG was examined at three different CO₂ levels. Inspiratory muscle activity in the sheep fetus is critically dependant on glutamate acting at non-N-methyl-D-aspartate (non-NMDA) receptors (Bissonnette et al., 1997). Reciprocal inhibition between inspiratory and expiratory neurons has been shown with intracellular recordings of membrane potential trajectories (see for example Fig 5 in (Bianchi et al., 1995)). We hypothesized that hypoxemic activation of expiratory activity results from the withdrawal of inspiratory neuronal inhibition and thus does not require glutamate neurotransmission. To test this hypothesis TA EMG activity was examined after instillation of the (R,S)- α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) glutamatergic receptor antagonist 2,3-dihydro-6-nitro-7-sulphamoyl-benzo(f) quinoxaline (NBQX) in the CSF of the fourth cerebral ventricle.

2. Methods

Surgical procedures

Sixteen pregnant ewes of mixed Western breed with known gestational dates were used for these experiments. All procedures and experiments were reviewed and approved by the Institutional Animal Care and Use Committee. Sterile surgery was carried out between 119 and 129 days gestational age (term is 147 days). The animals were premedicated with ketamine hydrochloride (3.0 mg kg⁻¹ i.v.) and diazepam (0.07 mg kg⁻¹ i.v.). Anesthesia was induced with sodium thiopental (21mg kg⁻¹) and maintained with inhaled halothane (0–5–1–5%) in nitrous oxide (50%) and oxygen (50%). The methods used for placement of catheters in the fetal trachea, carotid artery, fourth cerebral ventricle from a foramen magnum approach and amniotic fluid, and placements of electrodes above the dura and in the long muscle of the neck have been described previously (Bissonnette et al., 1995).

In addition for these studies electrodes were placed in the diaphragm (Di) and thyroarytenoid (TA) muscle. The posterior right Di was approached from an mid-axillary incision and the bared ends of Teflon-coated stainless-steel wire (As 633 F; Cooner Wire, Chatsworth, CA, USA) were sewn 1.0 cm apart into the posterior Di. Wires were placed in the right TA using a published method (Kianicka et al., 1994)

Protocols

The initial experiment was performed 4–5 days after surgical preparation of the fetus. Only a single experiment was performed on a given day and experiments were conducted while the fetuses were between 126 and 140 days old. Between one and five experimental protocols were performed in each fetus. All experiments were performed between 09.00 and 14.00 h. The trachea, amniotic fluid and arterial catheters were connected to pressure transducers (model 23 Db; Statham, Oxnard, CA, USA) that were calibrated with a mercury manometer. The leads for the fetal ECOG were connected to an amplifier and filtered (0.5–30 Hz

bandpass, type 9853A coupler; Beckman instruments, Schiller Park, IL, USA). The leads from the nuchal muscle, TA and Di were similarly connected, and the signals amplified and filtered (5.3–100 Hz). The signals from the transducers, ECoG and EMGs were displayed on a chart recorder (model R611; Beckman Instruments). The data were converted to digital form and stored for later analysis of the respiratory variables (model XT 286; IBM Instruments, Inc., Danburg, CT, USA). Fetal blood gases and pH were measured with a model 1306 pH/blood gas analyzer (Instrumentation Laboratories, Lexington, MA, USA) at 37°C and converted to 39°C.

NBQX was obtained from Tocris Cookson (Bristol, UK). NBQX was made up as concentrated stock in DMSO, and dilutions were made with Ringer solution (composition mequiv l⁻¹) Na⁺, 147.5; Cl⁻ 156; K⁺ 4.4; Ca⁺² 4.5; pH adjusted to 7.20 with NaOH) so that the desired concentration was always in a volume of 200 µl. The final concentration of DMSO was 1.25% for the NBQX injectate. Experiments with this concentration of DMSO in Ringer solution have shown no effect on breath amplitude or respiratory frequency nor on the duration of LV-ECoG episodes or the incidence of breathing in LV-ECoG (Bissonnette et al. 1997).

Data were continuously recorded for ~ 3–4 h. After 30 min of control, fetal arterial blood was sampled for Po₂, Pco₂ and pH. Following a 60 min control period hypoxic challenges were given for a 10 min trial. Fetal hypocapnic hypoxia was induced by having the ewe breath a mixture of 10% O₂ in nitrogen from a clear plastic bag. Blood gases were obtained midway through the hypoxia. For isocapnic hypoxia 4% CO₂/10% O₂ in nitrogen was used. Hypercapnic hypoxia was induced by pretreating the ewe with acetazolamide (200mg iv) and then breath 10% O₂. All hypoxic challenges were separated by a minimum of 30 min. In separate studies the AMPA receptor antagonist NBQX (100µg in 200µl Ringers) was instilled in the CSF of the fourth fetal cerebral ventricle by flushing the catheter with 500µl Ringers. Hypoxic trials were then repeated after 15 min. In order to determine the incidence of spontaneous tonic TA activity trials were conducted by having the ewe breath room air.

Analysis

Fetal breathing movements were defined as repeated transient negative pressures recorded from the trachea (relative to amniotic fluid pressure) that were greater than 1.0 mmHg and occurred at a frequency of greater than 0.25 Hz and lasted for a minimum of 15 s. All data are reported as means ± SD. Values for blood gases and pH during the control period were averaged for each fetus before obtaining a mean for the group. Relative TA activity for each hypoxic trial was obtained by normalizing the TA EMG during the trial to that in the pre-trial control period. Differences between the control and experimental protocols and between experimental protocols were examined with Fisher Exact Test when the incidence was less than 5 and Chi-square when it exceeded 5. pH and blood gas values were compared with repeated measures analysis of variance (ANOVA). P < 0.05 taken as a significant difference.

3. Results

Phasic activity in TA, confined to the early expiratory phase, was seen in 12 of 16 fetuses (Fig 1). These expiratory bursts in TA occurred during periods when breath frequency as seen in the Di EMG was relatively slow (29.9±8.0 bpm) and were not seen at higher frequencies (65.9±12.8 bpm).

Phasic TA muscle activity began immediately at the termination of Di activity and tracheal pressure deflection and ended well before the onset of the subsequent Di burst (Fig 1). Thus TA activity occurs during the post-inspiratory (early expiratory) (post-I) portion of the

breath cycle. During baseline studies prior to any hypoxic trials TA was often tonic during periods of Di inactivity associated with HV-ECoG. This was seen in 83.8% of 160 transitions from LV-ECoG to HV-ECoG in 15 fetuses.

Effect of hypoxia on TA activity

Prior to the hypoxic trial P_{O_2} values for the 3 carbon dioxide conditions were similar (range 19.2 to 21.0 torr) as was the level of subsequent hypoxia (11.9 to 14.2) (Table 1). The manipulations instituted to obtain graded CO_2 levels were effective: isocapnic P_{CO_2} , 53.2 ± 2.5 ; hypocapnic, 43.7 ± 0.6 and hypercapnic, 63.6 ± 2.9 ($P < 0.01$). Hypoxia induced tonic activity in TA (Fig 2). Tonic TA EMG was seen in 27 of 36 trials when CO_2 was kept constant (Table 2). The incidence of this tonic discharge was not affected by the concentration of CO_2 (Table 2). There were 29 (12 animals) hypoxic trials that were initiated when the fetus was in LV-ECoG. Examination of the temporal relationship between onset of tonic activity in TA and the switch to HV-ECoG showed that P_{CO_2} had no effect so the results were combined. In 20 trials the switch to HV-ECoG occurred after the onset of tonic TA 1.32 ± 0.89 min. There was no change to HV-ECoG in 3 trials, the switch was simultaneous in 3 and HV-ECoG preceded tonic TA (0.6 min) in 2. There was no tonic TA in 1 trial.

Effect of P_{CO_2} on the magnitude of hypoxia induced TA activity

The relative increase in TA activity was scaled by comparing thyroarytenoid EMG amplitude before hypoxia to that during the hypoxic trial. TA activity was inversely related to P_{CO_2} . In isocapnic hypoxia TA increased to 2.79 ± 1.51 fold above baseline; hypocapnic hypoxia, 4.0 ± 2.26 and hypercapnic hypoxia 2.21 ± 1.24 (Table 3). Regression of the data yielded: relative TA = $7.78 - (0.089 \times P_{CO_2})$ ($r^2 = 0.95$) ($P = < 0.05$).

Effect of AMPA blockade on hypoxic induced TA activity

As previously shown (Bissonnette et al. 1997), NBQX instilled in the 4th cerebral ventricle (100 μ g) abolished tracheal deflections and phasic Di activity without affecting the incidence of LV-ECoG. Fetal blood gas and pH values during hypoxia prior to NBQX treatment were not different from those after the drug (Table 3). The level of oxygen tension achieved was the same before and after instillation of NBQX (Table 3). AMPA receptor blockade did not affect the incidence of hypoxia induced tonic TA activity (Table 4).

4. Discussion

The principal findings of the present study are: 1) moderate hypoxia in fetal sheep is associated with tonic activity in the thyroarytenoid electromyogram, 2) this tonic TA activity precedes the switch from LV-ECoG to HV-ECoG, 3) the relative amplitude of hypoxia induced TA activity is inversely proportional to P_{CO_2} and 4) hypoxia associated TA activity persists after AMPA receptor blockade.

TA EMG activity during the baseline periods in this study was similar to that previously reported (Kianicka et al., 1998). During periods of relatively slow Di frequency TA was phasic with an onset immediate with Di discharge arrest and terminating well before the next Di burst. As previously suggested (St John et al., 1989) TA EMG most likely represents activity in post-I neurons. Kianicka and coauthors (1998) noted that continuous TA activity persisted when fetal breathing movements were absent during moderate hypoxia. They did not, however, systematically institute hypoxia shortly after the fetuses entered a LV-ECoG period and determine the temporal relationship between the onset of apnoea, tonic TA and switch to HV-ECoG. Here we show that tonic TA activity begins well before HV-ECoG in most cases. A common feature between the hypoxia trials and HV-ECoG during the

normoxic control periods is the absence of phasic inspiration. Thus it may be withdrawal of inspiratory inhibition of post-I neurons that effects tonic TA activity, rather than hypoxia itself. The present experiments do not allow a distinction between these two mechanisms.

The present results from fetuses in utero are similar to those in preterm lambs (Renolleau et al., 1999), but differ from findings in early postnatal (72 hours old) lambs (Praud et al., 1995). Renolleau and coauthors (1999) examined spontaneous apneas in lambs that were between 135 and 149 days post-conception. Lung maturation had been induced with steroid and thyrotropin-releasing hormone given to the ewe and labor was then induced at 131 days. TA was observed to be tonic during central apneas and during apneas associated with periodic breathing. These apneas were independent of behavioral state in these preterm animals. In contrast to the present report studies from the same group found no increase in TA EMG during induced hypoxia in term lambs at 72 hours of age (Praud et al., 1995). In the same animals during central apneas TA was tonic but the abdominal EMG was silent. This is consistent with post-I neurons being active while late-expiratory are inhibited. The result also points to tonic TA developing during withdrawal of inspiratory inhibition as suggested above. In the postnatal preterm lamb increased TA activity in central apneas would be beneficial in that lung volumes would be kept high allowing greater oxygen uptake. In the fetus where oxygen is of placental origin this does not apply. Nevertheless the TA EMG responses to cessation of inspiratory activity are in place in utero as they are in the preterm extra-uterine lamb.

The effect of P_{CO_2} on hypoxia induced TA activity is consistent with the concept that there is reciprocal activation and inhibition between expiratory and inspiratory neurons (Sears et al., 1982). TA activity showed its greatest increase over pre-hypoxic levels when the CO_2 was lowest. At low carbon dioxide inspiratory neuron activity would be low thus allowing greater activity in post-I neurons. The inhibition of tonic TA concurrent with Di activity (Fig 2) demonstrates post-I inhibition during inspiratory excitation.

Extracellular recordings from expiratory neurons in anaesthetized cats did not reveal that they fired tonically when hypoxia was induced (Richter et al., 1991), (England et al., 1995). Rather these neurons became silent and in one study (England et al., 1995) the decline in discharge preceded that in the phrenic motoneuron. The population of expiratory neurons in these studies, however, were late expiratory. As suggested above TA most probably represents post-I activity.

Thyroarytenoid motoneurons are located in the caudal aspect of the nucleus ambiguus (Pasaro et al., 1983), (Yoshida et al., 1982). Thus their excitatory synaptic input would be blocked by the 4th ventricle instillation of NBQX. The observation that hypoxia induced tonic TA activity persists after AMPA receptor blockade sufficient to silence Di EMG suggests that TA discharge results from withdrawal of inhibition.

In summary these results indicate that moderate hypoxia sufficient to cause apnea in fetal sheep is accompanied by tonic activity in TA EMG. Glutamate blockade at AMPA receptors does not affect this hypoxia induced TA activity which suggests that it results from withdrawal of inhibitory synaptic input.

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Figure 1. Representative traces during slow fetal breathing rate. Traces are from above: raw diaphragm EMG, integrated diaphragm EMG, tracheal pressure, raw thyroarytenoid (TA) EMG, integrated TA EMG, ECoG. Note that TA activity begins immediately when diaphragm ceases and ends well before the subsequent diaphragm burst.

Figure 2. Effect of hypoxia on thyroarytenoid (TA) EMG activity. Traces from above as in Fig 1. Record from 2 min 45 sec to 4 min 11 sec after onset of the hypoxic trial. Note in the right portion after diaphragm and trachea are silent their isolated bursts are associated with inhibition of TA EMG. Note that ECoG remains in low voltage during this period of hypoxia.

Table 1

Fetal blood gas values before and during hypoxia at three levels of CO₂.

Protocol	Control			Experimental		
	pH	P _{O2}	P _{CO2}	pH	P _{O2}	P _{CO2}
Isocapnic hypoxia	7.36±0.01	19.2±2.2	51.4±2.4	7.35±0.01	13.1 [*] ±0.5	53.2±2.5
Hypocapnic hypoxia	7.32±0.03	21.0±1.6	49.9±1.3	7.40 ^{**} ±0.01	11.9 [*] ±1.2	43.7 ^{**} ±0.6
Hypercapnic hypoxia	7.25 ^{**} ±0.02	20.5±2.7	65.6 ^{**} ±2.5	7.26 ^{**} ±0.01	14.2 [*] ±1.9	63.6 ^{**} ±2.9

Values are mean ± SD.

^{*} significantly different from control, $P < 0.01$ (ANOVA);

^{**} significantly different from isocapnic hypoxia, $P = < 0.05$.

The control blood samples for hypercapnic hypoxia were obtained after acetazolamide had been administered to the ewe.

Table 2

Effect of hypoxia on thyroarytenoid (TA) muscle activity in fetal sheep.

Protocol	Number of animals	Number of trials	Tonic TA	Incidence (%)
Room air	7	22	0	0
Isocapnic hypoxia	13	36	27	75*
Hypocapnic hypoxia	8	22	18	82*
Hypercapnic hypoxia	8	20	14	70*

* P<0.001 compared to room air.

The incidence of tonic TA did not differ between isocapnic, hypocapnic and hypercapnic hypoxia, P values between 0.816 and 1.000.

Table 3

Fetal pH and blood gases before and after NBQX.

Protocol	Control			Experimental		
	pH	P _{O2}	P _{CO2}	pH	P _{O2}	P _{CO2}
Hypoxia	7.35±0.02	18.2±1.0	52.1±1.0	7.33±0.02	12.8* ±2.4	53.9±3.0
NBQX then hypoxia	7.36±0.02	17.7±2.3	52.6±2.6	7.34±0.01	11.9* ±1.0	57.0±3.7

Values are mean ± SD.

* significantly different from control, $P < 0.05$ (ANOVA).

Table 4

Effect of AMPA receptor blockade on the incidence of hypoxia induced tonic TA activity.

Protocol	Number of animals	Number of trials	Tonic TA	Incidence (%)
Room air	7	22	0	0
Hypoxia	8	13	9	69*
NBQX then hypoxia	8	18	12	67**

* P=0.001,

** P=<0.001 compared to room air.

The incidence of tonic TA did not differ between hypoxia before NBQX and hypoxia after NBQX instillation in the 4th ventricle, P=1.000.