ARTICLES

Bronchial Muscle Peristaltic Activity in the Fetal Rat

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ABSTRACT: Aside for the potential for tonic contraction, the airway smooth muscle exhibits intermittent phasic rhythmic activity that may contribute to lung growth during fetal life. Therefore, we examined 4th generation rat 18–22 d gestation fetal, 4–6 d of age newborn and adult bronchial ring from Sprague Dawley rats to compare differences in smooth muscle function. We hypothesized that phasic contractions were greatest before birth. Bronchial muscle spontaneous rhythmic contractions were greatest in the fetus and absent in the adult. In response to KCl stimulation, the fetal bronchial smooth muscle only developed tonic force that was 3.5 ± 0.6 and lower than measured in the newborn 9.0 ± 0.3 and adult 13.7 ± 1.4 mN/mm². The thromboxane A₂ analogue U46619 induced tonic and phasic muscle contractions and the amplitude and frequency of the phasic contractions were greater in the fetus as compared with the adult and increased with gestational age. The U46619-induced rhythmic contractions were abrogated by ryanodine, thapsigargin and reduction of extracellular Na⁺, suggesting intracellular Ca²⁺ dependence and involvement of the Na⁺/Ca²⁺ exchanger. The inward rectifier K⁺ blocker BaCl₂ induced phasic contractions in unstimulated fetal, but not adult bronchial muscle of the same amplitude and frequency as for the spontaneous and U46619-induced ones. We conclude that the airway smooth muscle phasic activity is greatest in the fetus and tends to disappear post-natally with age suggesting an *in utero* role during lung development. (*Pediatr Res* 59: 756–761, 2006)

Although airway smooth muscle contraction is an integral part of the acute and chronic bronchoconstriction observed in conditions such as asthma, the role of this musculature in health is unclear (1). As opposed to the tonic muscle activity that determines the airway caliber and is responsible for bronchoconstriction, phasic spontaneous activity has been described in the trachea and smaller airway muscle of adult rabbits (2), humans (3,4) and guinea pigs (5). This phasic activity, however, is intermittent and not present in all airway smooth muscle *in vitro* preparations.

Recently, the existence of spontaneous bronchial muscle phasic activity in fetuses has been recognized (6,7). Its physiologic significance remains obscure, but McCray *et al.* (7), Schittny *et al.* (8), and more recently, Jesudason (9) suggested that such airway phasic contractions play an important role in

the maintenance of adequate alveolar distention. Yet others have raised objections to this speculation (10).

We reasoned that if the airway muscle phasic contractions are of importance to fetal lung growth this activity would be greatest before and progressively disappear after birth. Therefore the purpose of the present study was to compare phasic activity of airway muscle in fetal, neonatal and adult rats, as well as study the mechanism responsible for this oscillatory behavior.

Given that in fetal lung explants bronchial peristalsis was observed in small airways we studied near resistance fourth generation bronchi. In addition to recording their spontaneous activity the bronchial muscle was stimulated with voltage-dependent agonist KCl and the thromboxane A₂ analogue U46619. The latter agonist has been shown to induce both tonic and phasic contractions in certain smooth muscle containing tissues (11).

METHODS

Sprague-Dawley rats (Charles River, Saint-Constant, Quebec, Canada) were used for these studies. We evaluated 12 adults, 52 fetuses and 39 newborn animals. The fetuses were delivered at 18 ($n = 11$), 21 ($n = 3$) or 22 d ($n = 38$) gestation (term = 22 d) by cesarean section following pentobarbital sodium anesthesia (25 mg/kg IP). The newborn (4–6 d) and adult (2 mo) animals were killed with an overdose of pentobarbital sodium (50 mg/kg IP). All animals had their lungs removed immediately after death.

This research protocol was approved by the Hospital for Sick Children Animal Care Committee.

Organ bath studies. Fourth generation left lung intralobar bronchial rings (average diameter 70–100 μ m and length = 2 mm) were dissected free and mounted on a wire myograph (Danish Myo Technology A/S, Denmark). Isometric changes were digitized and recorded online (Mydaq, Danish Myo Technology A/S, Denmark). Tissues were bathed in Krebs-Henseleit buffer (NaCl, 115 mM; NaHCO₃, 25 mM; NaHPO₄, 1.38 mM; KCl, 2.51 mM; MgSO₄ · 7 H₂O, 2.46 mM; CaCl₂, 1.91 mM; and dextrose, 5.56 mM), bubbled with air/6% CO₂ and maintained at 37°C.

After 1 h of equilibration, the optimal resting tension of the tissue was determined by repeated stimulation with 128 mM KCl until maximum active tension was reached. All subsequent force measurements were obtained at optimal resting tension. Contractile responses were normalized to the tissue cross-sectional area as follows: (width × diameter) × 2 and expressed as mN/mm², as previously described (12).

Determination of bronchial contractility. KCl (128 mM prepared by equimolar substitution for NaCl in the Krebs-Henseleit solution) and the thromboxane A₂ analogue U46619 were used to evaluate the airway muscle

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tonic contraction. The bronchial muscle phasic activity was evaluated under optimal resting tension (spontaneous) and following stimulation with the thromboxane A₂ analogue U46619 at tonic EC₅₀ concentration (determined in preliminary studies to be 10⁻⁷ M).

To address the mechanism responsible for the bronchial phasic contractility, studies were also performed utilizing the non-specific cyclooxygenase blocker indomethacin (Sigma Chemical Co., Oakville, Ontario, Canada), the RYR inhibitor ryanodine (Calbiochem, San Diego, CA, USA), the sarcoplasmic reticulum Ca²⁺-ATPase (SERCA) pump inhibitor thapsigargin (Research Biochemicals International, Natick, MA, USA), the potassium channel blockers barium chloride, glibenclamide and 4-aminopyridine (Sigma Chemical Co., Oakville, Ontario, Canada) and the Rho-kinase inhibitor Y27632 (Calbiochem, San Diego, CA). All drugs were mixed in water and the bronchial contractility assessed for a period of 5 min before and after the addition of the drugs. A reduced-Na⁺ Krebs solution was prepared by equimolar replacement of NaCl with choline chloride (Sigma Chemical Co., Oakville, Ontario, Canada).

Statistical analysis. Statistical comparisons were done by either one- or two-way analysis of variance (ANOVA) or the *t*-test as indicated utilizing the Number Cruncher Statistical Package software (NCSS Kaysville, UT). Data are reported as Mean ± SE and a *p* < 0.05 was considered statistically significant.

RESULTS

Maturation differences in bronchial muscle tonic contraction. In response to KCl stimulation, the adult bronchial smooth muscle tonic active maximal force was 13.7 ± 1.4 and significantly higher (*p* < 0.01) than measured in the newborn 9.0 ± 0.3 and fetus 3.5 ± 0.6 mN/mm² (Fig. 1). A similar pattern of maturational differences in force development was observed in response to U46619 stimulation.

Maturation differences in bronchial muscle phasic contractions. Representative tracings of the fetal, newborn and adult bronchial muscle spontaneous phasic contractions are shown in Fig. 2. The magnitude and pattern of phasic contractions were age-dependent, but in contrast with the tonic force of greater amplitude and frequency in the fetal and newborn airways, as compared with the adult.

Given that the spontaneous phasic activity of the fetus and newborn was not regular and continuous, we sought to consistently reproduce it *via* stimulation with the thromboxane A₂ analogue U46619, as used by others (13). U46619 EC₅₀ (10⁻⁷ M) induced phasic activity was only present in the fetal and newborn bronchial muscle in a pattern similar to the one

Figure 1. Twenty-two days gestation fetal (○, *n* = 3), newborn (◐, *n* = 6) and adult (●, *n* = 12) bronchial muscle response to KCl stimulation. ** *p* < 0.01 versus fetal and newborn values by two-way analysis of variance.

Figure 2. Representative tracings of thromboxane A₂ analogue U46619-induced tonic and phasic contractions and unstimulated spontaneous phasic activity of fetal, newborn and adult bronchial smooth muscle. Note that the rhythmic phasic activity is almost absent in the adult and of greatest magnitude in the fetus in the spontaneous and U46619-induced contractions

observed for the spontaneous contractions (Fig. 2). No phasic contractions were seen at any age in response to KCl stimulation.

Figure 3 illustrates the amplitude and frequency of the bronchial muscle phasic contractions as a percentage of U46619-induced force. The phasic contractions amplitude was significantly higher in the fetus, as compared with the newborn and adult, whereas the frequency was similar in fetal and newborn tissue but significantly different from adults. As illustrated in Fig. 4, the phasic activity expressed as amplitude × frequency of the fetal bronchial muscle significantly increased (*p* < 0.01) as gestation progressed and was highest at term.

Role of prostaglandins and rho-kinase. To further evaluate the mechanism responsible for the bronchial muscle phasic activity, all studies were done utilizing fetal bronchi stimulated with U46619 EC₅₀ (10⁻⁷ M).

Given the developmental changes in lung prostaglandin levels and their unique role in fetal life such as the maintenance of ductus arteriosus patency we investigated the effect of a cyclooxygenase inhibitor upon the bronchial phasic activity. Indomethacin (10⁻⁵ M) had no effect on the amplitude and frequency of the U46619-induced fetal phasic contractions, suggesting no involvement of cyclooxygenase products in this process.

Figure 3. Amplitude (expressed as percentage of the active force) and frequency of the U46619-induced (10⁻⁷ M) bronchial muscle phasic contraction in 22 days gestation fetal (*n* = 20), newborn (*n* = 12) and adult (*n* = 5) tissue. ** *p* < 0.01 vs adult; †† *p* < 0.01 versus newborn by one-way ANOVA and Tukey-Kramer multiple comparison test.

Figure 4. Bronchial muscle peristaltic activity represented as the product of amplitude $\times$ frequency of U46619-induced (10^{-7} M) from 18 ($n = 11$), 21 ($n = 3$) and 22 d gestation ($n = 3$) fetal rats. ** $p < 0.01$ versus 22 d; †† $p < 0.01$ versus 21 d by one-way ANOVA and Tukey-Kramer multiple comparison test.

We further investigated whether Rho-kinase had played a role inducing phasic activity *via* increased Ca^{2+} sensitization. The rationale for this set of experiments related to our recently published data showing that pulmonary arterial smooth muscle Rho-kinase activity is highest in the fetus (14) and the observation by others that this enzyme is involved in embryonic organogenesis (15). Therefore we investigated the effect of Y27632, a Rho-kinase inhibitor, on the U46619-induced fetal bronchial phasic contractions. Progressively increased Y27632 molar concentration (10^{-9} to 10^{-6} M) resulted in a significant decrease of the U46619-induced tonic contraction from 2.7 ± 0.1 mN to 1.9 ± 0.1 mN at 10^{-6} M of Y27632 ($p < 0.01$). Yet the U46619-induced phasic contraction amplitude was 0.9 ± 0.1 before and 0.7 ± 0.1 mN in the presence of Y27632 10^{-6} M concentration ($n = 4$; $p = \text{NS}$) suggesting that rho-kinase activity is not responsible for the phasic activity of the fetal bronchial muscle.

Role of extra and intracellular Ca^{2+} pools. Calcium free media and the Ca^{2+} chelator EGTA resulted in complete suppression of the U46619-induced tonic, as well as phasic bronchial muscle contractions indicating that both are dependent on the extracellular calcium concentration.

To investigate the role of intracellular calcium pools we used the RyR channel inhibitor ryanodine and the SERCA pump inhibitor thapsigargin. Ryanodine ($10 \mu\text{M}$) selectively diminished the U46619-stimulated fetal bronchi rhythmic activity frequency with no effect on the tonic component of the contractions (Fig. 5). Thapsigargin (10^{-6} M) significantly ($p < 0.01$) reduced the frequency, but not amplitude of the phasic contractions (Fig. 6) Together these data suggest involvement of the RyR and thapsigargin-sensitive Ca^{2+} stores in the mechanism of phasic contractions.

Contribution of potassium channel activity to the phasic contractions. Membrane ion channels play an important role in smooth muscle excitability and K^{+} channels are known to play an important role in the resting potential of airway smooth muscle (16). For this reason we tested the effect of K^{+} channel blockade on the fetal bronchial muscle U46619-induced phasic contractions. The K_{ATP} inhibitor gliben-

Figure 5. Amplitude and frequency of the U46619-induced (10^{-7} M) bronchial muscle phasic response in fetal bronchial muscle ($n = 3$) in the absence (control) and presence of ryanodine ($10 \mu\text{M}$). ** $p < 0.01$ versus control by paired t -test.

Figure 6. Amplitude and frequency of the U46619-induced (10^{-7} M) bronchial muscle phasic response in fetal bronchial muscle ($n = 4$) in the absence (control) and presence of thapsigargin (10^{-6} M). * $p < 0.05$ versus control by paired t -test.

clamide (10^{-5} M) had no effect on the amplitude (1.2 ± 0.2 versus 1.4 ± 0.14 mN) or frequency (6.5 ± 0.85 versus 7.8 ± 0.6 contractions/min) as compared with the control U46619-induced bronchial muscle phasic contractions ($n = 4$). Similar lack of effect was observed with the K_v channel blocker 4 aminopyridine (10^{-5} M) where the amplitude was 0.26 ± 0.05 versus 0.16 ± 0.04 and the frequency 7.7 ± 1.8 versus 7.8 ± 1 as compared with control values ($n = 4$).

Addition of the inward rectifier K^{+} current inhibitor BaCl_2 (10^{-3} M) had no effect on the amplitude and frequency of U46619-induced bronchial muscle phasic contraction in the fetus. Yet, when fetal bronchial rings were exposed to BaCl_2 (10^{-3} M) without U46619 stimulation, an immediate and persistent phasic muscle activity was observed (Fig. 7). The amplitude and frequency of the BaCl_2 -induced contractions were comparable with the spontaneous and U46619-induced rhythmic activity. Exposure of adult bronchial muscle to BaCl_2 (10^{-3} M) did not induce phasic or tonic contractions.

$\text{Na}^{+}/\text{Ca}^{2+}$ and $\text{Na}^{+}/\text{H}^{+}$ exchange and phasic contractions. The potential role of $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger in the fetal bronchial muscle U46619-induced phasic contractions was investigated because thoracic duct smooth muscle peristaltic activity is dependent on this channel (11). The fetal bronchial muscle U46619-induced phasic contractions amplitude was

Figure 7. Amplitude and frequency of the fetal bronchial spontaneous muscle phasic response ($n = 4$) in the absence (control) and presence of barium chloride (10^{-3} M). ** $p < 0.01$ versus control by paired t -test.

significantly ($p < 0.01$) abrogated in sodium-reduced buffer (Fig. 8) suggesting involvement of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. To exclude the possibility of such a suppressive response being related to the Na^+/H^+ channel activity, we evaluated the effect of and inhibitor of this channel (amiloride) on the U46619-induced peristaltic motion. The amplitude of fetal bronchial muscle ($n = 4$) phasic activity following amiloride (10^{-4} M) treatment was 0.6 ± 0.2 and not significantly different from control 0.4 ± 0.05 mN). Similarly, amiloride had no effect on the U46619-induced frequency of phasic contraction in the fetal bronchial muscle (amiloride = 5.8 ± 1.85 versus control = 8.5 ± 2.6 contractions/min).

DISCUSSION

Aside from its mainly tonic force generation the airway muscle also exhibits a phasic like activity (4). In 1924, Lewis (17) was the first to report spontaneous airway contractility in chick embryos with a frequency of 2 contractions/min. Airway muscle phasic activity has been subsequently reported by others to be present in the postmortem tissue from adult (4,18,19) and fetal humans (7), as well as in some animal species (2,20,21). Such phasic activity is not unique to the

airway and is also present in lymphatic vessels (22), the thoracic duct (11), systemic arteries (13) and bladder smooth muscle (23).

In the adult human and animal tissue the airway muscle phasic activity is intermittent and not always present (2–4). In the present study we observed the highest spontaneous and U46619-induced bronchial muscle activity in the fetal rat at term with an average frequency of 10 contractions/min. The U46619-stimulated rhythmic contractions increased with gestational age *in utero* and were of maximal activity at term. Following birth, these phasic contractions decreased and were absent in the adult rat bronchi. The pattern observed for the phasic contractions contrasts with the age-dependent increase in tonic force development of the bronchial muscle in the rat.

Although the present study involved bronchial ring segments studied *in vitro*, the fetal airway muscle rhythmic activity under these conditions was similar to the one reported by Schittny in freshly isolated pig and rabbit, as well as cultured mouse fetal lungs (8). The use of the thromboxane A_2 agonist U46619 to stimulate phasic contractions in this study was necessary to induce consistent and reproducible phasic activity to allow further evaluation of its mechanism. Aside from its powerful smooth muscle tonic contraction response, U46619 has been shown by others to induce phasic activity in the thoracic duct (11), coronary arteries (24), and dog lingual and mesenteric arteries (25). Of interest is the fact that following KCl stimulation the fetal bronchial muscle develops tonic force without phasic contractions suggesting that the mechanism by which U46619 induces rhythmic activity does not depend on the tonic force.

The mechanism responsible for the airway muscle phasic activity in the fetus is presently unclear, but available data indicate the following. It is independent of the airway innervation since atropine and tetrodotoxin do not suppress such phasic activity (7). Calcium channel blockade with nifedipine halted airway rhythmic contractility in the murine explant (26) and human lung (7). In the embryonic rat lung peristaltic airway activity was dependent on extracellular calcium entry via L-type voltage-gated and intracellular Ca^{2+} stores (27).

In the present study we sought to further evaluate the mechanism involved in the spontaneous contractions by studying the U46619-induced phasic contractions. That this thromboxane A_2 analogue-induced phasic contractions mimic the spontaneous ones is best illustrated by the similarities between the present study utilizing U46619 and Featherstone *et al.* data on the spontaneous peristaltic waves of the fetal rat airway (27). In both preparations the phasic activity was suppressed by removal of extracellular calcium and blockade of SERCA and RyR regulated internal calcium stores.

We have demonstrated in the present study that the bronchial muscle rhythmic contractions were also abrogated by a reduction of extracellular Na^+ and amiloride at concentrations known to inhibit Na^+/H^+ exchange did not affect the phasic contractions. Together these findings suggest that the fetal bronchial muscle phasic activity depend on the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Similar findings of $\text{Na}^+/\text{Ca}^{2+}$ involvement in U46619-stimulated rhythmic smooth muscle contractions have been reported for the pig thoracic duct (11). Coupling of

Figure 8. Amplitude and frequency of the U46619-induced (10^{-7} M) bronchial muscle phasic response in fetal bronchial muscle ($n = 3$) in Krebs-Henseleit (control) and sodium free buffer. ** $p < 0.01$ versus control by paired t -test.

the $\text{Na}^+/\text{Ca}^{2+}$ exchanger with Ca^{2+} release from intracellular stores has been shown in the guinea pig ileal smooth muscle (28) and a similar mechanism may be involved in the fetal rat airway phasic activity.

We further evaluated the potential role of potassium currents in the phasic activity of the fetal bronchial muscle. The four classes of K^+ channels known to exist in the airway smooth muscle include the Ca^{2+} -dependent (K_{Ca}), voltage-dependent (K_{V}), ATP-dependent (K_{ATP}) and inward rectifier (K_{IR}) (29). Inhibition of K_{Ca} channels in the fetal rat lung did not significantly alter the amplitude of peristaltic activity (27). In this study we found a similar lack of effect on fetal bronchial muscle U46619-induced phasic contractions amplitude and frequency when exposed to the K_{V} inhibitor 4-aminopyridine and the K_{ATP} blocker glibenclamide. Yet the K_{IR} inhibitor BaCl_2 induced spontaneous contractions in the fetal, but not adult bronchial muscle. The BaCl_2 -induced peristaltic activity was of similar amplitude and frequency that of the U46619 stimulated.

K_{IR} current was once believed to be non existent in airway smooth muscle (29), yet recently Snetkov *et al.* documented its existence in human small (but not large) airway smooth muscle (16). In addition, Snetkov *et al.* studying fetal human airway smooth muscle cells documented a strong similarity between these and their adult equivalent (30). In freshly isolated fetal cells a non-contractile proliferative phenotype is observed in 50%, as opposed to the adult human airway smooth muscle where almost all cells are contractile. When adult human bronchial smooth muscle cells are placed in culture and become proliferative a significant number of them exhibit inward rectifying K^+ current (31). Taken together these data suggest that K_{IR} channels are only existent in bronchial smooth muscle cells during fetal life and may have an important role in the mechanism accounting for airway peristaltic activity prenatally.

The possible role of Rho-kinase-induced Ca^{2+} sensitization on the mechanism accounting for bronchial muscle phasic activity was also investigated. It is widely known that muscle contraction is not only the result of an increase in intracellular Ca^{2+} concentration, but also Ca^{2+} sensitization (32). U46619 induces smooth muscle contraction in certain tissues by Ca^{2+} sensitization in a manner that is reversible by the Rho-kinase specific inhibitor Y27632 (33–35). To evaluate the Rho-kinase pathway involvement in the U46619-induced fetal bronchial peristaltic activity we progressively increased the Y27632 concentration of pre-stimulated bronchial muscle until a significant reduction in tonic force was observed. No change in the amplitude of phasic contraction was noted in the fetal bronchial muscle suggesting that this activity is not Ca^{2+} sensitization mediated.

A cartoon illustrating the site of action of all tested compounds with an effect on the tonic and phasic contraction are shown in Fig. 9. Based on the evidence of the present study and previously published observations, we speculate that the mechanism responsible for the bronchial muscle peristaltic activity is as follows. Cyclical inactivation of bronchial smooth muscle membrane K_{IR} (mostly present in the fetus) causes membrane potential depolarization *via* activation of

Figure 9. Illustrative cartoon of the site of action of the agonists and antagonist used to evaluate the mechanism responsible for the fetal bronchial phasic contractions. RyR, ryanodine receptor; U46619, thromboxane A_2 analogue; SERCA, sarcoplasmic reticulum Ca^{2+} -ATPase; IP $_3$ R, inositol triphosphate receptor; VOCC, voltage operated calcium channel; TP, thromboxane receptor

L-type VOCC Ca^{2+} channels. This result in an influx of extracellular Ca^{2+} and activation of SERCA and RyR pumps leading to depletion and refilling of intracellular Ca^{2+} stores. This together with Ca^{2+} extrusion from the intracellular compartment *via* the $\text{Na}^+/\text{Ca}^{2+}$ exchanger result in the oscillatory phasic behavior of the fetal bronchial muscle.

Lung growth is dependent on expansion of peripheral airspaces and related to the fluid pressure in the lungs during gestation (36). This pressure is presumed to be caused by secretion of liquid into the lung lumen and the occlusion of the airways at the level of the glottis. Aside from the presence of adequate amounts of lung fluid, fetal breathing leads to further distention of the alveoli. This rhythmic activity is thought to have an important role in lung growth, since fetal breathing suppression by phrenic nerve section leads to lung hypoplasia (37,38). Aside from breathing the fetal airway smooth muscle peristaltic activity may also play an important role toward lung growth. In fact, the only suggested physiologic function of airway muscle relates to its potential role during fetal life on the promotion of lung growth (8,9) leading to the speculation that post-natally the bronchial muscle is merely an appendix of the lung (1).

Whether the regulation of fetal breathing and bronchial contractility follows a similar pathway is unknown. Fetal breathing in humans increases with gestational age and is greatest near term (39). Schittny *et al.* reported consistent airway muscle phasic activity as early as 11 to 12 d in the fetal mice, 25 to 56 d in the fetal pigs and 27 d gestation in rabbits with the frequency of contractions late in gestation ranging from 2 to 12/minute (8). In the rat, periods of continuous fetal breathing are only detected on day 18th of gestation and progressively increase in duration until term (40). In the present study we demonstrated that the rhythmic activity of fetal bronchial muscle progressively increases after 18 d and is maximal at 22 d gestation in the rat, averaging 8/minute. Together these data suggest that the fetal breathing and bron-

chial rhythmic phasic activity follow the same *in utero* maturational pattern, possibly because of their complementary role in the promotion of lung growth.

In summary, spontaneously observed, BaCl₂-mediated and thromboxane A₂ analogue-induced rhythmic contractions are of greatest amplitude in the fetal airways and likely related to the unique characteristics of the fetal airway smooth muscle cells. The predominance of bronchial rhythmic phasic activity during fetal life supports the speculation that the airway muscle plays an important role toward lung growth before birth.

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