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# Acetylcholine Muscarinic Receptor-Mediated Contractility of Ovine Pulmonary Arteries: Changes with Maturation and Chronic Hypoxia

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# ACETYLCHOLINE MUSCARINIC RECEPTOR-MEDIATED CONTRACTILITY OF OVINE PULMONARY ARTERIES: CHANGES WITH MATURATION AND CHRONIC HYPOXIA

By
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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

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Reader: Dr. Anthony Verlangieri

**DEDICATION**

**To my parents, who through countless sacrifice gave me countless opportunities.**

## ACKNOWLEDGMENTS

I would like to thank Dr. Sean Michael Wilson for providing me with the opportunity to work with and be exposed to great minds. Without his fervor, dedication, unyielding patience, and wondrous generosity, this project would never have come into fruition.

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## ABSTRACT

Dan Hu Nguyen: Acetylcholine Receptor Mediated-Contractility in Ovine Pulmonary Arteries: Changes Due to Maturation and Chronic Hypoxia
(Under the Direction of Dr. Sean Michael Wilson)

Pulmonary vasculature tone is mediated by Acetylcholine Muscarinic Receptors (mAChR) located on the vascular smooth muscle wall which are responsible for vasoconstriction through the activation of $Ca^{2+}$ dependant intracellular signaling pathways and by the endothelium which is responsible for vasodilation through the generation of Nitric Oxide (NO). The disruption and subsequent loss of the endothelium results in vasoconstriction of pulmonary arteries (PA). This effect is observed in several studies utilizing different animal models.

Chronic Hypoxia (CH) has been shown to disrupt the endothelium which in turn alleviates the vasodilatory response associated with acetylcholine (ACh) stimulation. The interplay between smooth muscle myocytes and the endothelium allow for the hypothesis that CH reduces ACh-dependent PA contractility was tested by performing experiments using wire-myography in conjunction with endothelium-denuded PA rings from normoxic fetal and adult animals. The data indicates that ACh (100 μM) causes a decrease in tension in CH adults and completely ablated tension in CH fetuses of pre-constricted arteries via 125 mM KCl. The CH adult showed contraction when stimulated with 10 μM Carbachol (CCh) which is a selective muscarinic agonist. The tension generated both from ACh and CCh were completely ablated by 1 μM Atropine which is a selective $M_3$ muscarnic receptor subtype antagonist. Muscarinic receptor activation, as stated above, generates $Ca^{2+}$ dependant intracellular signaling. Confocal imaging *in situ* further substantiates the finding that mAChR activation initiates intracellular signaling

via $Ca^{2+}$ dependant pathways by showing a marked increase in cytosolic $Ca^{2+}$ levels after activation of mAChRs.

A notable finding is that there is no contractility in CH fetuses. The loss of the endothelium suggests that there should be vasoconstriction. However, this was not observed and the compensatory mechanism has not been elucidated. Perhaps there could be down-regulation of the mAChRs of the CH fetus in an attempt to match ventilation and perfusion rates in an attempt to maximize oxygen intake. The data garnered from this experiment could further facilitate other avenues of research in the hopes that therapeutic treatments for debilitating ailments such as Persistent Pulmonary Hypertension of Neonates (PPHN) can be discovered and or refined.

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| | |
|---|---|
| ACh | acetylcholine |
| AChE | acetylcholinesterase |
| ANOVA | analysis of variance |
| ATP | adenosine Tri-phosphate |
| $Ca^{2+}$ | calcium |
| CH | chronic hypoxia |
| $CO_2$ | carbon dioxide |
| ChAT | choline acetyl transferase |
| DAG | di-acetyl-glycerol |
| $IP_3$ | inositol 1,4,5 triphosphate |
| mAChR | acetylcholine muscarinic receptor |
| NO | nitric oxide |
| $O_2$ | oxygen |
| PA | pulmonary artery |
| PASMC | pulmonary arterial smooth muscle cell |
| PLC | phospholipase C |
| PKC | protein kinase C |
| PPHN | persistent pulmonary hypertension of the newborn |
| ROCK | rhoa-dependant kinase |
| SR | sarcoplasmic reticulum |
| SEM | standard error of mean |
| Ve/Q | perfusion to ventilation ratio |

## Introduction

L. Frank Baum was once quoted as saying "Whenever I feel blue, I start breathing again." Breathing clearly serves a much higher purpose than to make people happy but without oxygen one could neither be happy nor sad. Cellular metabolism requires the use of potent electron acceptors in aerobic respiration to generate ATP. The lungs are critical to this process as they provide a medium for gaseous exchange between the internal and external environments.

People are negative pressure breathers, which means when inhalation occurs, the pressure in the lungs is less than the atmospheric pressure. The pressure difference, and simple diffusion, then provides the driving force for inhalation. The pressure gradient is established by the increase in the volume of the lungs by the chest wall and thoracic cavity through contraction of the diaphragm and intercostal muscles. The increased volume results in a decrease in pressure within the alveoli, which is in accordance to Boyle's Law. The high atmospheric pressure coupled with a low intra-alveolar pressure results in air flowing down its pressure gradient. The alveoli, small membranous sacks surrounded by capillaries, are the site of gaseous exchange within the lungs. The gases exchanged are $O_2$, for aerobic respiration and $CO_2$, a metabolic byproduct of $O_2$ and glucose metabolism.

The vessels associated with the lungs are responsible for taking $O_2$ from the lung to the systemic vasculature and out to the periphery and for delivering $CO_2$ from the working tissues to the lung. Thus, a primary goal of the lung is to match ventilation to perfusion. Airway contractility controls the resistance of air movement in and out of the

lung while pulmonary vasoreactivity regulates blood flow. The contractility of airways and vessels are regulated by the nervous system, endocrine system and local mediators.

Local control of pulmonary vessels comes in several forms. First and foremost, the arteries of the lung are unique in that they constrict in response to tissue hypoxia, as opposed to the system vasculature whose vessels relax when the oxygen tension falls. Akin to the systemic vasculature the endothelium is known to release both vasoconstricting agents such as endothelin and vasodilatory modulators such as Nitric Oxide, which until recently was regarded as nothing more than a passive barrier lining the lumen of blood vessels (Shimoda *et al, 2000).* The endothelium releases and responds to vasoactive mediators, hormonal and humoral substances and to physical stressors such as stretching or shearing of the muscle wall (Shimoda *et al, 2000*; Altiere, *et al, 1994*). Neural and hormonal innervation of the pulmonary vasculature rests with the release of select hormones and neurotransmitters. Neural control is primarily associated with the release of norepinephrine through sympathetic innervations, which causes vasoconstriction (Shimoda *et al,* 2000). The binding of norepinephrine to the α-1 adrenergic receptor, which is found on most-all smooth muscle, causes vasoconstriction (Taylor *et al,* 2008). The β-2 adrenergic receptor is responsible for vasodilatory innervation by the sympathetic nervous system (Kou *et al, 2007*). The release of serotonin from neuroepithelial bodies in the lung as well as circulating platelets causes membrane depolarization of smooth muscle cells through activation of G-protein coupled receptors and associated intracellular signaling pathways (Kupchik *et al,* 2008). Classically, acetylcholine is thought to be a vasodilatory substance through its activation of muscarinic receptors on the endothelium, which ultimately causes NO generation

(Shimoda *et al,* 2000). However, several reports show that acetylcholine can cause vasoconstriction in the pulmonary vasculature (Shimoda *et al,* 2000; Altier *et al,* 1994 ).

Research indicates that parasympathetic innervation of pulmonary airways is mediated through the release and uptake of acetylcholine (ACh) (Shimoda *et al 2000*). Yet, less is known about the actions of ACh on the pulmonary vasculature. In general, muscarinic receptors on smooth muscle, bind ACh and their activation is responsible for smooth muscle contraction to this neurotransmitter. Empirical evidence has shown that $M_1$ and $M_3$ muscarinic receptors may be located on pulmonary arterial (Altiere *et al,* 1994) and airway smooth muscle cells. These $M_1$ and $M_3$ receptors are Gq-protein coupled receptors, which mediate their intracellular effects through calcium dependent and independent pathways. Ultimately, the role of acetylcholine as a vasoactive agent is dependent on its production, release, uptake, removal and activation of several intracellular signaling cascades.

Typically, acetylcholine production begins in the parasympathetic neuron innervating the smooth muscle. Production of acetylcholine is mediated by the enzyme choline acetyl transferase (ChAT), which catalyzes the binding of acetate and choline. The ACh molecules are packaged in vesicles, which then await release. The transmission of a nervous impulse along the axon reaches the nerve terminal, which then results in the release of acetylcholine into the synaptic cleft. Once present in the synaptic cleft, ACh will then bind onto Muscarinic receptors on the postsynaptic membrane.

Once bound, acetylcholine activates the receptor, which initiates several intracellular signaling cascades. In particular, there is activation of phospholipase C (PLC) as well as RhoA dependent pathways (Shafer *et al, 2004*). PLC gives rise to

Inositol 1,4,5 triphosphate ($IP_3$)and di-acetyl-glycerol. The IP3 initiates release of $Ca^{2+}$ from the sarcoplasmic reticulum (SR) through activation of IP3 receptors while DAG activates Protein Kinase C (PKC). In addition, there is activation of extracellular $Ca^{2+}$ influx through L-type $Ca^{2+}$ channels and non-selective cation channels. The RhoA dependent pathway activates Rho-dependent Kinase (ROCK). Together, the calcium and kinase dependent signaling pathways increase smooth muscle contractility. The bound acetylcholine is degraded by the enzyme acetylcholinesterase (AChE) to acetate and choline. This degradation of ACh then inactivates the acetylcholine response in pulmonary arterial smooth muscle.

The endothelium is implicated in the generation of nitric oxide, which in turn causes a vasodilatory response in the systemic and pulmonary vasculatures, a process that is also dependent on muscarinic receptor activation by ACh. Chronic hypoxia is known to disrupt the endothelium of both pulmonary and systemic vasculature (Shimoda *et al,* 2000; Altier *et al,* 1994). Disruption of the endothelium in systemic vasculature results in neither vasoconstriction nor vasodilation when stimulated with acetylcholine as arterial smooth muscle cells of most vascular beds do not express muscarinic receptors. However, stimulation with acetylcholine of disrupted endothelium in pulmonary vasculature results in "paradoxical" vasoconstriction, which we hypothesize will be observable in pulmonary arteries isolated from fetal and adult sheep. Some of this work has appeared in abstract form (Nguyen *et.al*, 2008).

## Methods

### Animals and Arterial Dissection

The lung samples were obtained from Loma Linda University. The lungs were isolated and shipped on ice to the University of Mississippi, arriving ~ 24 hours after animal sacrifice. The lungs were obtained from both healthy normoxic and hypoxic sheep of either sex of age 18-24 months. The fetal lung samples were obtained from fetuses who were of 139-141 days of gestation and near term. The fetuses were also separated into hypoxic and normoxic groups. The hypoxic set was exposed to hypoxia in the womb through their mothers who were subjected to hypoxic conditions. To induce hypoxia, animals were housed at the White Station research facility (12,500 feet). When transported to Loma Linda University, the hypoxic animals were maintained in a hypoxic state by performing a tracheotomy and ventilating them with a gas mixture that simulates the oxygen environment at the white mountain research station. The animals were euthanized with 100 mg/kg sodium pentobarbital intravenously. The lung tissue was placed into plastic storage containers filled with physiological buffered saline solution for shipping. Upon arrival, the lung samples were removed from the storage container and placed into a dissection dish filled with ice-cold Krebs – hasseleit (K-H) buffer of the following composition in mM: 120 NaCl; 4.8 KCl; 1.2 $K_2HPO_4$; 25$NaHCO_3$; 1.2 $MgCl_2$; 2.5$CaCl_2$; 5 Glucose Intrapulmonary arteries with internal diameters of 800 μm - 1.5 mm were isolated from the lungs, cut into 5 mm long segments, and stored on ice until use.

**Contractility Measurements**

The arterial rings were mounted on wires with one end being fixed and the other being attached to a low impedance force transducer (Radnoti Glass, Monrovia CA) and lowered into a 5 ml bath chamber containing K-H buffer bubbled with 95% $O_2$ and 5% $CO_2$, which mimics fully oxygenated blood and warmed to 37C.

To see if acetylcholine was responsible for eliciting a myogenic response, pulmonary arteries were constricted with a high potassium (High-K) solution where all sodium in the K-H solution was replaced in equimolar fashion for potassium. This results in a solution that had 125 mM K, which effectively depolarizes the cellular membranes of the smooth muscle cells to 0 mV, and thus activates L-type $Ca^{2+}$ channels as well as other voltage-dependent ion channels. Specifically, the bath was emptied of K-H solution and replaced with 125 mM K at 37 °C. The artery was given approximately 20-30 minutes to fully contract and plateau. Once the arteries were maximally contracted, 100 μM Acetylcholine was added. This procedure was repeated for all four sets of animals: fetal and adult normoxic, and fetal and adult hypoxic.

**Confocal Microscopy**

The isolated arteries that were denuded of endothelium were loaded with 10 μM Fluo-4 and 0.1% pluronic F127 for 1 to 1.5 hours at room temperature in the dark. The tissue was then washed with a balanced salt solution buffered with HEPES for 30 min to allow for dye esterification. The arterial segments were then cut and mounted on a sylgard block placed in an open bath imaging chamber. The images were obtained through a confocal microscope (Zeiss 510 META) by exciting Fluo-4 with a 488 nm argon laser and collecting emitted light at wavelengths greater than 510 nm. Twelve bit

Images were acquired (512 X 512 pixels) at ~ 1 Hz using bi-directional scanning techniques and individual myocytes were examined post-hoc for changes in fluorescence in the absence and presence of acetylcholine.

**Statistics**

A t-test or a two-way analysis of variance (ANOVA) with a bonferroni post-hoc analysis was performed when appropriate. A $P<0.05$ was considered significant. All values expressed were mean ± standard error of the mean (SEM).

**Reagents and solutions**

Most all reagents were purchased from Sigma Aldrich (St. Louis, MO), with the exception of DAU 5884, Pirenzipine, and Atropine which were purchased from Tocris (Ellisville, MO).

## Results

Figure 1 displays the average tension generated by stimulation with 100 μM acetylcholine after full membrane depolarization of the cellular membrane with high-K. Accordingly, the y-axis measures the percent increase in contractility after the initial contraction induced by high K ($\%T_{Kmax}$.) The fetal normoxic and adult normoxic displayed approximately the same percent (35%) of increase in tension relative to $T_{Kmax}$. Most notably, vessels from hypoxic fetuses did not contract in response to Ach, while vessels from adult hypoxic animals may not contract as well as to Ach as their normoxic counterparts. Based on a 2 x 2 analysis of variance (maturation X hypoxia) the data show there is an interaction between maturation and hypoxia on the ability of acetylcholine to cause pulmonary arterial tension. Particularly, chronic hypoxia causes a marked reduction in the tension developed by 100 μM ACh only in pulmonary arteries from fetuses that were exposed to chronic hypoxia. The fetal normoxic displayed 36 ± 9 % (mean ± SEM) in 18 arteries, which was the same as adult normoxic that was 36 ± 5 % in 26 arteries. Vessels from adult hypoxic sheep responded less well being 19 ± 3% in 24 arteries of the high-K contraction while those from hypoxic fetuses did not contract in response to Ach, being - 4 ± 3% in 16 arteries.

We then evaluated the ability of acetylcholine to contract unstimulated pulmonary arteries isolated from hypoxic fetuses. This is shown in figure 2 where the tension developed in response to 100 μM acetylcholine is shown. The tension, measured in grams, was nearly identical to the force generated in the absence of ACh, where the tension was 0.57 ± 0.02 g for 35 arteries in the absence of Ach and 0.59 ± 0.02 g in the

presence of 100 μM Ach. The data set was obtained from the same set of arteries used to generate figure 3.

Figure 3 shows the change in tension in response to 100 μM acetylcholine in unstimulated pulmonary arteries. In comparison to arteries preconstricted with high-K, vessels from adult hypoxic animals demonstrated the greatest amount of tension development. We are unsure of why there are differences in the response to ACh between high-K preconstricted arteries (Figure 1) and those that were previously unstimulated (Figure 3) but potentially there may be a role for activation of L-type $Ca^{2+}$ channels by Ach as occurs with serotonin in these vessels. Notably, the Ach dependent force development was nearly identical in vessels from adult and fetal normoxic animals, mimicking the findings shown in figure 1. Also similar to that of figure 1 is the finding that vessels from fetal hypoxic animals display little to no contractility to Ach, which as depicted in figure 2. Based on a 2-way analysis of variance (maturation X oxygenation), vessels from chronically hypoxic fetuses are less responsive to ACh than those isolated from hypoxic adult. Further to this, there is an interactive effect where the vessels from hypoxic fetus are also less reactive to ACh than those isolated from normoxic fetus or adult.

Figure 4 shows a representative tracing of arterial tension over time during the addition of 10 μM carbachol, a selective muscarinic agonist in a pulmonary artery from a hypoxic adult. Carbachol application caused a rise in vascular tone of 0.25 g, from 0.50 to 0.75 g. Once the contraction plateaued, 1 μM atropine, a selective muscarinic antagonist was added to the bath. The resulting decrease in contractility is evident as the

effect was immediate and vascular tone drops from greater than 0.75 g to less than 0.50 g, which is below baseline.

Figure 5 shows the average data based on results from experiments performed on arteries from adult hypoxic animals such as that depicted in figure 4. The figure shows that pulmonary arterial smooth muscle cells of adult hypoxic animals have functional muscarinic receptors. The tension generated by 100 μM acetylcholine was nearly nullified by 1 μM Atropine. The force generated by 10 μM carbachol was approximately 0.2 g, being comparable to that generated by 100 μM acetylcholine. Importantly, 1 μM atropine also reduced the tension developed by 10 μM carbachol, providing further support for a role of muscarinic receptors.

On average Ach caused a contraction of 0.23 ± 0.04 g while atropine depressed this substantially, being 0.02 ± 0.009 g ($P<0.001$ by a paired t-test). These data were collected on 4 experimental days in 11 arteries. Replicating these experiments with carbachol did not influence the experimental findings where CCh alone caused contractions of the same magnitude, being 0.29 ± 0.03 g in 4 arteries over 2 days, while atropine reduced the tension to 0.015 ± 0.02 g.

Figure 6 shows the arterial tension over time in a representative artery in response to 100 μM carbachol and the influence of 10 μM Dau 5884, a relatively selective M3 receptor inhibitor. The figure illustrates that the tension generated by CCh, approximately 0.8 grams of force, was completely ablated by 10 μM of Dau 5884, where the tension after inhibition by Dau 5884 dropped below basal levels.

Figure 7 shows summarized data for the influence of $M_1$ (pirenzipine, PIR) or $M_3$ (Dau 5884) selective muscarinic receptor inhibitors on Ach or CCh developed tension in

vessels isolated from adult normoxic or hypoxic sheep. Dau 5884 caused a dramatic decrease in tension as force dropped below 100% in the normoxic adult. Selective $M_3$ inhibition with 10 μM Dau 5884 also caused a significant decrease in the tension developed in the hypoxic adult. Pirenzipine was far less effective at blocking carbachol mediated contractility, as its effect on the hypoxic adult was negligible. Overall, 10 μM Dau 5884 caused contractility in response to CCh to be depressed by 136 ± 21% in 14 arteries from 3 normoxic adult animals. In adult hypoxic animals, Ach-mediated contraction was reduced by 57 ± 2% in 3 arteries from 1 animal. Pirenzipine reduced CCh contraction by 33 ± 1% in 4 arteries from one normoxic adult animal while in 2 arteries from a single hypoxic adult PIR reduced Ach contraction by 15 ± 5%.

Notably proper controls have not been performed to determine if the modest effect of PIR is due to the action of the drug or of an artifact due to vasorelaxation over time. Because there can be vasorelaxation of the arteries in the continued presence of muscarinic receptor stimulation more rigorous experimentation is required to substantiate the findings of figure 7. Specifically, a schild analysis of the effects of various inhibitors of $M_1$, $M_2$ and $M_3$ receptors on ACh or CCh induced contractility would provide more comprehensive and conclusive information regarding the relative role to the contractility responses shown in these studies.

Figure 8 shows calcium signaling events in an arterial segment from a hypoxic adult that was exposed to 100 μM acetylcholine. Prior to ACh there was little or no $Ca^{2+}$ activity. ACh stimulation caused varied $Ca^{2+}$ responses depending on the cell examined. Many cells did not exhibit any $Ca^{2+}$ responses. However, a number of cells, such as those shown in Figure 8B, showed $Ca^{2+}$ responses. Most of the responding cells showed

multiple $Ca^{2+}$ spikes in response in ACh administration, which is consistent with activation of $M_1$ or $M_3$ muscarinic receptors.

## Discussion

The purpose of this study was to investigate the effects of chronic hypoxia and maturation on muscarinic mediated contractility in pulmonary arteries. Indeed, our studies confirm earlier reports showing paradoxical contraction of pulmonary arteries through activation of muscarinic acetylcholine receptors (Altiere *et al,* 1994). More importantly, the present studies expand on this earlier work and show that high altitude exposure causes a loss of acetylcholine-mediated contractility in fetus. What is more, the work indicates that these changes are not simply due to postnatal maturation as muscarinic receptor induced contractility is well-developed in arteries of fetuses from ewes raised at low altitude.

Our data provide evidence that acetylcholine causes pulmonary arterial contractility through the activation of muscarinic receptors and not nicotinic receptors. This is important, as nicotinic receptors are ligand gated ion channels while muscarinic receptors are linked to intracellular signaling cascades (Sherwood, 2007). In particular, carbachol, a selective muscarinic receptor agonist, induces pulmonary arterial contractility, while atropine, a selective muscarinic receptor antagonist inhibits Ach and CCh reactivity. Second to this, muscarinic-dependent contractility is $Ca^{2+}$ dependent, as evidenced by our confocal imaging studies and is most likely due to $M_3$-receptor activation as the contractility response is inhibited by Dau 5884. Notably, there may also be a more limited role for $M_1$-receptor activation as PIR inhibited a portion of the contractility response. This latter effect will be the subject of future investigations.

Although acetylcholine-mediated contractility is dependent on cytosolic $Ca^{2+}$ increases, it appears to be independent of membrane depolarization. Evidence for this is based on the finding that acetycholine induces contractility in arteries that were prestimulated with 125 mM KCl. This high concentration of potassium depolarizes the myocyte membrane to 0 mV. This depolarization fully activates L-type $Ca^{2+}$ channels as well as other voltage-dependent ion channels. The additional tension generated by acetylcholine therefore supports the idea that the contractility response to acetylcholine can proceed through pathways independent from changes in membrane potential although the data are suggestive that depolarization dependent pathways may be important in arteries from hypoxic adults.

The lack of contractility differences between fetal and adult normoxic vessels indicates that muscarinic pathways are well-developed before birth. However, there is an interactive effect of hypoxia on vessels from preterm sheep, as they show little to no Ach-dependent contractile activity. What is more, vessels from normoxic fetus and adult as well as hypoxic adult display almost identical tension development, and thus would alter lung blood flow similarly. The lack of Ach-mediated reactivity in vessels from fetal hypoxic sheep indicates high altitude causes undue stress on the unborn fetus.

The compensatory mechanism responsible for the lack of Ach-dependent constriction in pulmonary arteries from hypoxic fetuses is unknown. Potentially, mAChR contractility may act to fine-tune blood flow so that ventilation and perfusion can be well-matched. The reduced oxygen tension in the fetus may lead to loss of this fine-tuned control pathway so that perfusion is maximized at the expense of the Ve/Q ratio, which would allow for the most efficient extraction of oxygen.

Persistant Pulmonary Hypertension of the Newborn (PPHN) is a condition in which the fetus is unable to adapt its lung physiology for oxygen intake due to the inability of the vessels to dilate and thus decrease vascular resistance (Bernbaum *et al, 1984).* The resistance is often high enough to divert blood flow from the pulmonary to the systemic vasculature through shunts such as the foramen ovale. The blood shunted through these channels travels to the systemic vasculature without being properly oxygenated. Chronic hypoxia is shown to disrupt the endothelium, which is responsible for the generation of nitric oxide and vasodilatory responses in the pulmonary vasculature. Without the endothelium, we should expect to see vasoconstriction due to the presence of the M receptors located on the smooth muscle cells. However, in vessels from hypoxic fetuses, there is no response and thus there is neither vasoconstriction nor vasodilation. The lack of constriction also indicates that there may be down-regulation of muscarinic receptors, which are normally expressed. From a physiological perspective, it is likely that the fetus is attempting to compensate for the low oxygen tension by eliminating mechanisms responsible for diverting blood flow in response to tissue hypoxia. When the animal is born this inhibition of vasoconstriction may then serve as a protective role acting to maximize oxygen uptake.

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## Figures

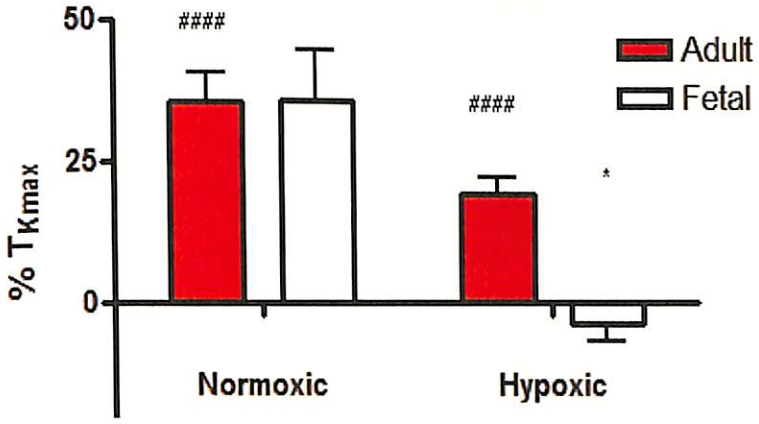


**Figure 1:** The tension ($\%T_{Kmax}$) generated by 100 µM ACh in fetal and adult normoxic are almost identical, but hypoxia depresses tension development. Bars denote mean ± SEM for adult normoxic (n=26), fetal normoxic (n=18), adult hypoxic (=24), fetal hypoxic (n=16). There is no tension generated in the fetal hypoxic. Based on a 2-way ANOVA, #### Denotes the effect of hypoxia as compared to normoxic controls ($P<0.0001$). * Denotes the influence of maturation relative to its control ($P<0.05$).

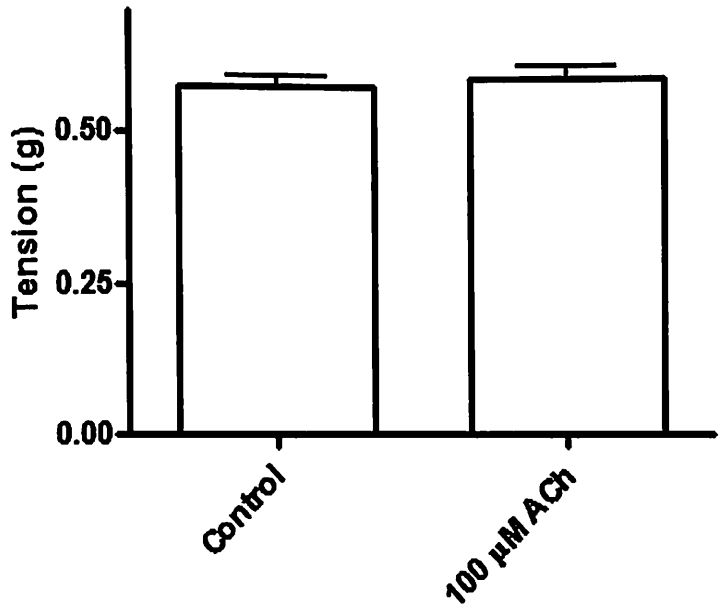


**Figure 2:** Average raw tension of stimulation with 100 µM Ach as compared to controls. Bars denote ± SEM for control (n=35) and 100 µM Ach in the same arteries. No differences were found by a paired t-test.

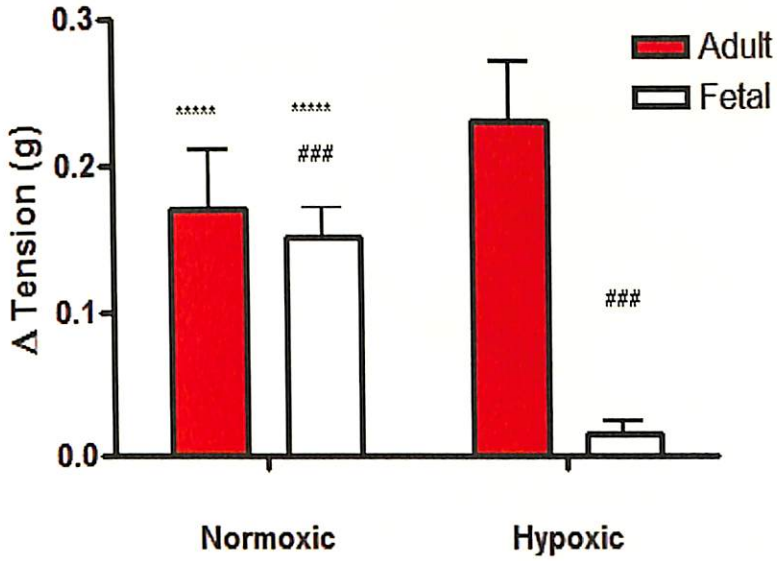


**Figure 3:** The tension developed by 100 mM ACh is nearly identical among the vessels examined except for arterial segments from hypoxic fetus. Bars denote ± SEM. Based on a 2-way ANOVA, differences were noted based on maturation ($P<0.0001$) as denoted by ***** and an interactive effect of maturation and chronic hypoxia ($P<0.001$) as denoted by ###.

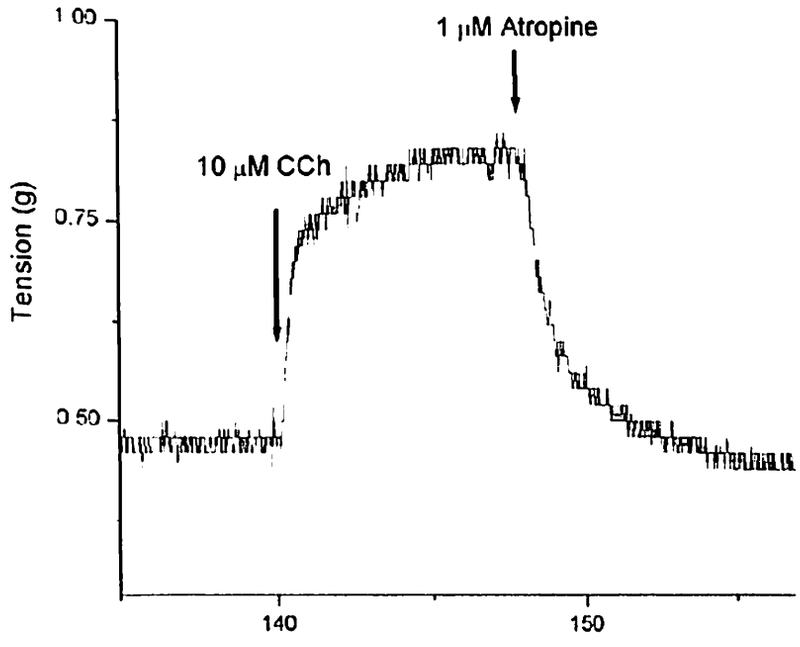


**Figure 4:** Representative trace of pulmonary arterial tension over time showing that stimulation with CCh causes a rise in tension while atropine causes tension to drop below basal levels.

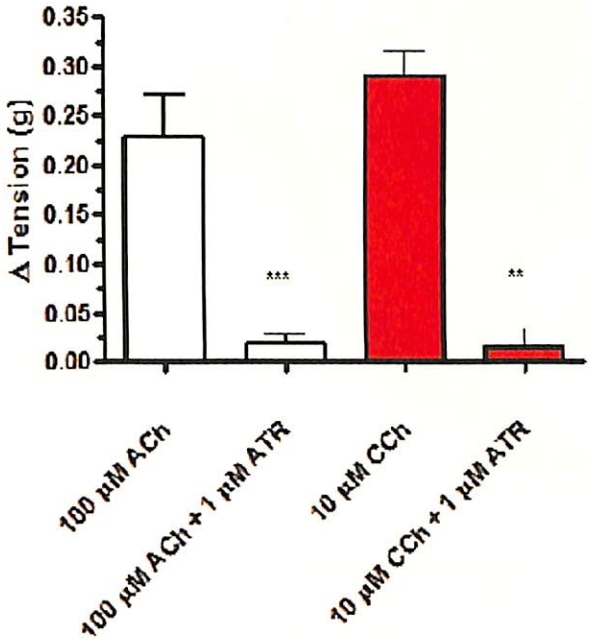


**Figure 5:** Atropine inhibits the tension generated due to stimulation with Ach and CCh in pulmonary arteries from hypoxic adult sheep. Bars represent ± SEM. Ach was tested on 11 arteries while CCh in 4 arterial segments. Statistical differences by t-test analysis are denoted by ** ($P<0.01$) and *** ($P<0.001$) relative to Ach or CCh.

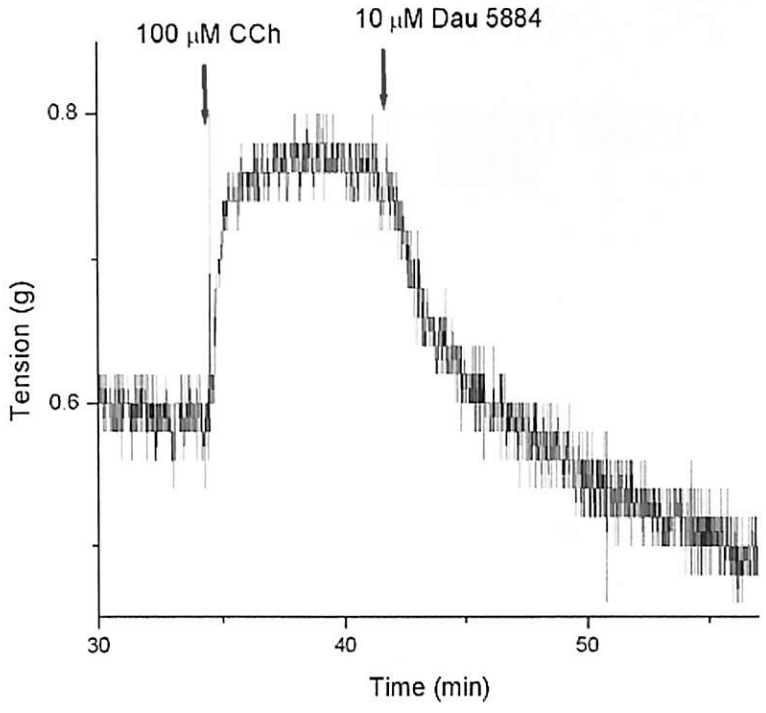


**Figure 6:** Representative tracing of adult normoxic pulmonary arterial tension over time. Stimulation with CCh causes a rise in tension to approximately 0.8 g while DAU 5884, a selective $M_3$ antagonist, completely ablates tension.

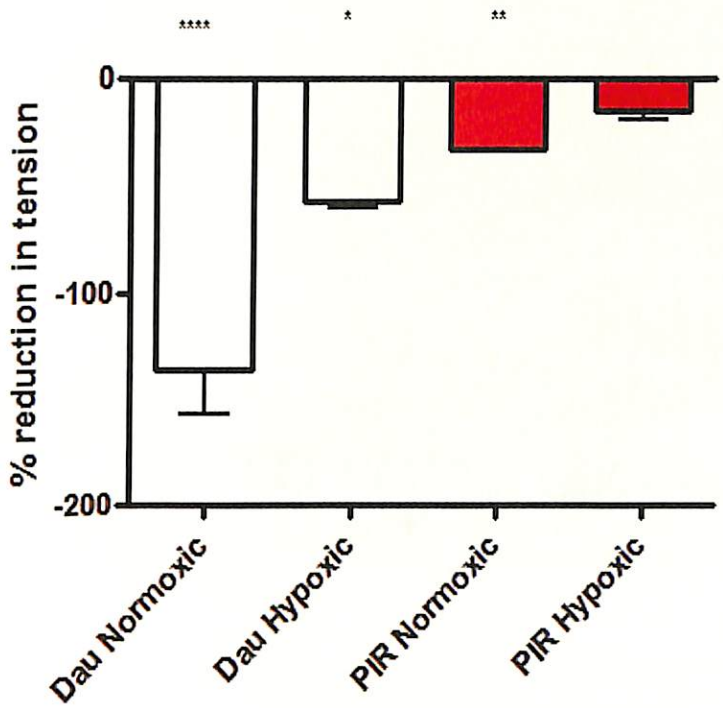


**Figure 7:** DAU 5884 depressed pulmonary arterial tension due to acetycholine receptor activation while Pirenzipine (PIR) had little effect. Bars denote ± SEM for the effects of Dau on Normoxic (n=14) and hypoxic (n=3), and PIR on normoxic (n=4). There were insufficient samples to perform statistical analyses on the effects of PIR in vessels from hypoxic animals (n=2). **** denotes $P<0.0001$, ** $P<0.01$ and * $P<0.05$ for a paired t-test analysis for the effects of drug on contractility in response to Ach or CCh.

A B

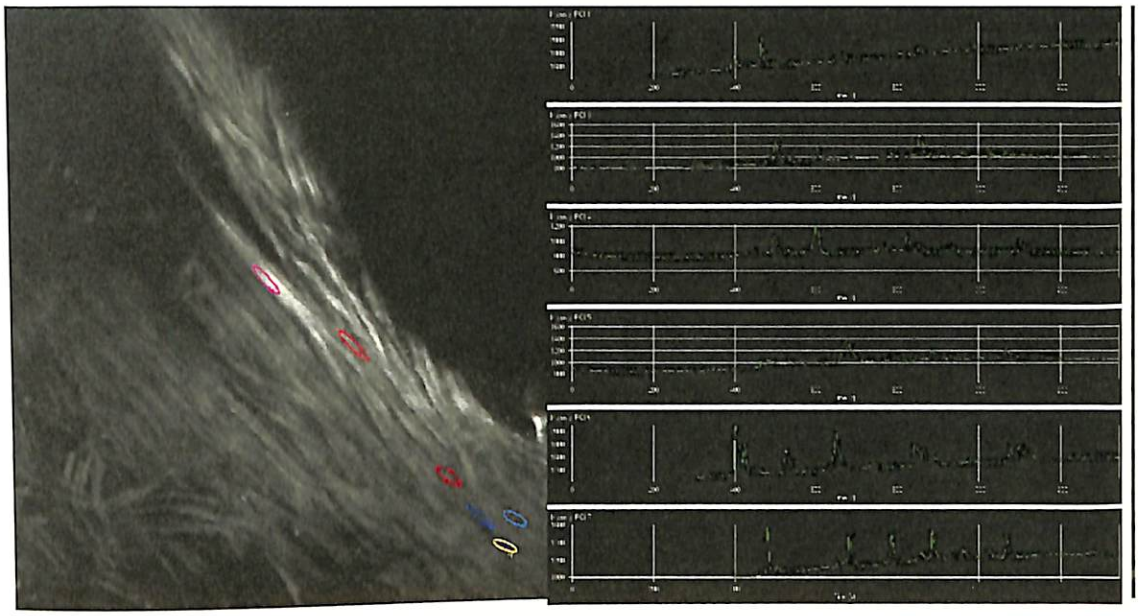

**Figure 8:** 100 µM acetycholine induced $Ca^{2+}$ responses in pulmonary arterial myocytes *in-situ* from a hypoxic adult. (A) Fluorescence of Fluo-4 for a pulmonary arterial segment from a hypoxic adult and regions of interest analyzed changes in fluorescence over time (B).