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Intimations on the Development of Effective Treatment for the Prevention of Preterm Birth: β3 Adrenergic Receptor Signaling in the Human Myometrium

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Abstract

Preterm birth before the thirty-seventh week of pregnancy is the leading cause of infant morbidity and mortality in the US. Infants born prematurely can spend weeks or months in the hospital, costing upwards of thirty billion a year in the US alone. Treatments for patients in labor preterm are ineffective and none are FDA approved. The β3 adrenergic receptor (β3AR) has been shown to be present in various tissues, including the human myometrium. Using contractile studies, stimulation of the β3AR has been shown to mediate relaxation. Although some pathways have been described, the mechanism(s) underlying β3AR-mediated relaxation in the myometrium are incompletely known. In other smooth muscles, the β3AR has been shown to mediate relaxation *via* nitric oxide (NO)-guanylyl cyclase-cGMP signaling. However, NO-mediated relaxation of the myometrium is cGMP-independent. Our studies showed that β3AR agonist, mirabegron, can be used to mediate relaxation in the human myometrium. The mechanisms associated with this relaxation are revealed to be increased production of NO in myometrial endothelial cells, stimulation of Ca^{2+} activated K^+ channel (BK_{Ca}), and down regulation of the contractile associated protein connexin 43 (Cx43). The mechanosensitive channel Piezo1 was identified in the myometrium and showed to promote relaxation using similar mechanisms involving NO and $BK_{Ca.}$ Our studies showed that NO mediated S-nitrosation of Cx43 promote a hemichannel state over a gap junction state. Together this research indicates the potential of β3AR as a target for developing tocolytic strategies involving combination therapy with mechanisms that have additive effects.

Dedications

To Iain Buxton, for giving me the opportunity to change my life

To Humza and Misha Asif, for never giving up on me

To Hina Warsi, my love and my best friend

To my parents, Abdus and Riffat Asif, for their infinite love and support

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Chapter 1

Introduction: Preterm Labor and the pursuit of effective tocolytic strategies.

Introduction:

Background of Preterm Labor

Preterm labor (PTL) occurs when a pregnant woman has sustained uterine contractions prior to 37 weeks of gestation. PTL is subcategorized as early or late preterm, with early PTL occurring at or before 33 weeks and late PTL occurring between 34 and 36 weeks (Suman and Luther 2021). PTL is a major global public health issue about 10% of pregnant women in the United States go through PTL (Martin et al. 2021). Although PTL still affects many women, despite the abundant amount of research performed, there are no effective answers to how and why spontaneous PTL occurs and what should be done to treat it (Haas et al. 2009; Olson et al. 2008).

There are several risk factors linked to preterm labor, including infections such as chorioamnionitis, smoking or the use of illicit drugs, mothers being of advanced maternal age (>35 years old), ethnicity of the mother, polyhydramnios, and chronic maternal conditions such as high blood pressure or diabetes (Purisch and Gyamfi-Bannerman 2017). African American women have a 50% greater risk of experiencing a preterm birth than Caucasian women (Bryant et al. 2020). Furthermore, certain social determinants of health put women at much higher risk for PTL, including stress, access to prenatal care, and food insecurity (Dolatian,

Sharifi, and Mahmoodi 2018). Although there are several risk factors associated with PTL, spontaneous PTL accounts for approximately half of PTL cases.

The earlier the delivery, the more likely the infant will face complications such as blindness, respiratory distress syndrome, jaundice, infections, and brain hemorrhaging (Ward and Beachy 2003). Along with these complications, other long-term issues may arise as well, such as chronic lung disease and neurodevelopmental disabilities. While there are no FDA-approved treatments for preterm labor, current non FDA-approved treatments for patients in PTL are largely ineffective and are only able to stall labor for a maximum of 48 hours (Reinebrant et al. 2015; Crowther et al. 2014; Flenady et al. 2014; Neilson et al. 2014). In 2005, the economic burden in the United States associated with PTL, combined with its associated complications, cost approximately $26.2 billion dollars, and this annual sum has only continued to grow through the ensuing years (Boardman 2008).

Although 10% of all pregnancies in the United States are classified as preterm (Martin et al. 2021), other countries with poor reproductive health initiatives have even higher incidences of PTL and associated adverse outcomes. In sub-Saharan Africa, approximately 28% of all newborns do not survive (Kinney et al. 2010). In Pakistan, poor reproductive health has continued to affect the PTL and neonatal mortality rates. The Global Network Maternal Newborn Health Registry is an observational study that includes all pregnancies and related outcomes recorded in India, Pakistan, the Democratic Republic of Congo, Guatemala, Kenya, and Zambia. In this study, the still birth rate in Pakistan was 53.5 per 1000 births compared to the 23.5 per 100 births average of the other

countries (Aziz et al., 2020). A cross-sectional study shows that the prevalence of PTL in Pakistan is about 21%, which greatly contributes to infant mortality (Hanif et al. 2020). These rates of neonate morbidity and mortality in other regions of the world emphasize just how critical it is to identify treatments for PTL.

The smooth muscle of the uterus, the myometrium, is the source of contractions in laboring women; therefore, pursuing research into understanding how it functions and what we can target to promote a state of relaxation is worthwhile (Egarter and Husslein 1992). Here, I will discuss the background of PTL, the pathophysiology of a myometrium in a contractile and quiescent state, and current ineffective tocolytic strategies for treating PTL. We investigate novel tocolytic strategies involving the β3 adrenergic receptor, and the multiple downstream pathways associated with it.

Myometrium

The myometrium is the smooth muscle of the uterus where uniform contractions are produced during labor. The myometrium lies between the endometrium and perimetrium, which together make up the uterine wall. The myometrium is composed of two layers. The circular layer is thinner and is located at the innermost aspect of the muscle fibers. This layer is derived from the paramesonephric/Mullerian ducts (McEvoy and Tetrokalashvili 2020). The outer longitudinal layer is made up of highly vascularized, muscle bundles in an extracellular matrix made of collagen which promotes contractile strength, and is

derived from non-Mullerian tissue (McEvoy and Tetrokalashvili 2020). Understanding the physiology of how contraction and relaxation works in the myometrium is necessary to identify different mechanisms to target for treating PTL.

Similar to skeletal muscle, the myometrium requires depolarization and an increased intracellular Ca^{2+} concentration in order to contract. This contraction is limited by the number of ATP available; however, the myometrium is not necessarily limited by the amount of ATP available (Pham and Puckett 2020). The process by which the myometrium changes from a quiescent to a contractile state encompasses numerous factors. First, the myometrium is unique compared to other smooth muscle systems. Smooth muscle found in the digestive tract uses enteric neurons to stimulate contractions, while vascular and pulmonary smooth muscle are heavily affected by adrenergic stimulation (Nezami and Srinivasan 2010; Barnes 1995). The myometrium, on the other hand, can be depolarized and have an increase in Ca^{2+} influx through hormone receptor activation (i.e., oxytocin and estrogen receptors) and proinflammatory factors (Peltier 2003).

Oxytocin receptors are members of the G-protein-coupled receptor subfamily, and they initiate the G_q pathway. When oxytocin binds to the receptor, it activates phospholipase C, which then hydrolyzes phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-triphosphate and diacylglycerol. Diacyglycerol activates protein kinase C, and inositol 1,4,5-triphosphate releases calcium from the sarcoplasmic reticulum (Vrachnis et al. 2011). Also, activation of the oxytocin receptor is believed to induce membrane depolarization and promote calcium

influx through voltage-dependent calcium channels; however, the specific mechanism is not yet known (Ferreira et al. 2019). The Ca^{2+} then binds to calmodulin, which allows it to interact with myosin light chain kinase. Once myosin light chain kinase is activated, it proceeds to phosphorylate myosin light chain at serine residue 19, which leads to the myosin cross-bridge interacting with actin filaments to induce contractions (McEvoy and Tetrokalashvili 2020). Thus, smooth muscle, such as the myometrium, can maintain an ongoing state of contraction or relaxation based off phosphorylation events (Hong et al. 2011). To inhibit this interaction, myosin phosphatase removes the phosphate and halts contractions (Hudson and Bernal 2012).

Myosin phosphatase is not activated when it is phosphorylated on the myosin phosphatase target subunit 1 at the threonine 696 site (Seko et al. 2003). Oxytocin-induced activation of protein kinase C (PKC) promotes different signaling cascades to stimulate an increase in prostaglandins, and PKC interacts with CPI-17 (Ozaki et al. 2003; Vrachnis et al. 2011). Phosphorylated CPI-17 inhibits myosin phosphatase from increasing the myosin cross-bridge interaction with actin, which ultimately leads to a more profound contractile effect (Ozaki et al. 2003). Prostaglandins mediate cervical ripening, which softens and opens the cervix to allow the baby to pass through during labor (Ivanišević, Djelmiš, and Buković 2001). Prostaglandins also increase the contractile effect of the myometrium by increasing oxytocin and upregulating intercellular communication (Ivanišević, Djelmiš, and Buković 2001).

Unlike the skeletal muscle, the myometrium utilizes intercellular communication through the formation of gap junctions, formed by connexin 43, which plays a critical role in propagating contractions through Ca^{2+} exchange in the myometrium (Döring et al. 2006). The state of labor has been shown to be associated with an increase in connexin 43 gene expression (Lye 1996). Connexin 43 channel activity can be modulated through different phosphorylation events. Connexin 43 channel activity can be mediated by phosphorylation on the serine 364/365 (Lampe et al. 2000). The disassembly of the connexin 43 gap junctions can be mediated by different phosphorylation sites including serine 368 and tyrosine 247/265 (Solan and Lampe 2014; Lampe et al. 2000). Disassembly of the connexin 43 gap junction leads to a decrease in intercellular communication to favor relaxation and formation of hemichannels, which then has the potential to release small molecules, such as ATP and prostaglandin E2 (Kang et al. 2008; Burra and Jiang 2009). Previous studies have shown that the release of ATP and prostaglandin E2 by hemichannels is partly due to the mechanical strain the cell faces (Genetos et al. 2007; Cherian et al. 2005).

In order to maintain quiescence during gestation, the myometrium relies on progesterone to inhibit the production of several contractile-associated proteins (Zakar and Mesiano 2011). During labor, however, women do not experience a significant decrease in progesterone until after the delivery of the placenta (Feinshtein et al. 2010). This functional progesterone withdrawal may be due to the catabolism of progesterone into inactive compounds or modulation of the progesterone receptor itself (Brown, Leite, and Strauss 2004). Also,

antiprogesterone agents, such as RU486 (mifepristone), are used to induce medical abortions or manage miscarriages (Cadepond et al.1997)Thus, another molecule must be considered when aiming to relax a laboring uterus.

Nitric oxide is considered a major mediator of smooth muscle relaxation. During pregnancy, nitric oxide is produced in the myometrium, decidua, and placenta (Norman and Cameron 1996). In smooth muscle, nitric oxide usually interacts with soluble guanylyl cyclase to produce cyclic GMP, which then activates protein kinase G, which then activates the myosin phosphatase to relax the smooth muscle (Francis et al. 2010). In the myometrium, however, nitric oxide causes relaxation in a cGMP-independent manner (Karri K Bradley et al. 1998). Nitric oxide has the capability to bind to glutathione to make S-nitrosoglutathione, and this compound has the ability to carry nitric oxide and attach it to a cysteine thiol of a protein in a process called S-nitrosation (Ulrich et al. 2013). Studies have shown that proteins essential to myometrial contraction are capable of being S-nitrosated (Barnett et al. 2018; Ulrich et al. 2013). Although S-nitrosoglutathione promotes relaxation in the myometrium in PTL tissue, the effect becomes blunted due to the increased expression of S-nitrosoglutathione reductase (Scott D. Barnett et al. 2018). We investigated pathways involving nitric oxide production along with other quiescent factors that have an additive effect to promote relaxation.

Current Tocolytic Strategies

Tocolytics are drugs that are used to delay or prevent uterine contractions. With PTL as a global public health issue, effective tocolytic strategies need to be developed. With labor potentially being associated with a multitude of processes involving hormones, different immunological factors, infections, and stress, it gives researchers options to target various areas when developing tocolytics. One of the first tocolytic drugs was the hormone relaxin. The physiology of relaxin was not completely understood when it was first proposed as a tocolytic; however, researchers knew it was present in serum from women within the first trimester and that it promoted quiescent effects in rats (Abramson and Reid 1955). In a clinical trial, relaxin was administered to fifty patients who elected to be induced into labor, and relaxin had no significant effect on delaying labor (Babcock and Peterson 1959). Afterwards, other drugs were pursued to determine their effectiveness as tocolytics.

Most drugs tested for their viability as tocolytics were initially designed for other purposes. Atosiban, however, was specifically developed to be a tocolytic. Atosiban is a synthetic peptide which works as a competitive antagonist against oxytocin at the oxytocin receptor (Fullerton et al. 2011). As mentioned earlier, oxytocin promotes a positive feedback loop of contractile effects by increasing intracellular calcium concentration and prostaglandin activity. Atosiban has been shown to inhibit prostaglandin-induced contractile pathways as well (Kim et al.

2019). Although it seems like the most appropriate choice for treating PTL, clinical studies have shown it to be significantly ineffective alone (Fullerton et al. 2011).

Calcium channel blockers are the most commonly used drugs for tocolytics (Haas et al. 2012). As Ca^{2+} is the prime second messengers in promoting contractions, inhibiting channels that increase the intracellular concentration of Ca^{2+} makes sense. Calcium channel blockers were originally developed to treat patients with hypertension by preventing the tonic contractions of the vascular smooth muscle. Two of the most common calcium channel blockers used are nifedipine and magnesium sulfate. Although calcium channel blockers currently have the highest probability of delaying PTL, the delay only lasts up to 48 hours (Haas et al. 2012). Prolonged treatment with calcium channel blockers can lead to adverse effects such as tachycardia, hypotension, and even hypocalcemia (Holmes et al. 1994; Malha and August 2019; Koontz et al. 2004).

As prostaglandins play a significant role in promoting the positive feedback loop to perpetuate contraction, studies investigated the efficacy of their inhibitors as possible tocolytics. Non-steroidal anti-inflammatory drugs, such as sulindac and nimesulide, have been tested, but the most commonly prescribed tocolytic in this class is indomethacin (Loudon et al. 2003). Along with promoting contractile effects, prostaglandins prevent the closure of the fetal ductus arteriosus. The ductus arteriosus allows oxygenated blood to bypass the pulmonary system to flow directly into systemic circulation (Huff and Mahajan 2019). The duct usually closes spontaneously after birth; however, if it does not close, then indomethacin can be administered to help it close (Pacifici 2013).This not closing phenomenon is known

as patent ductus arteriosus. Some complications that may arise from patent ductus arteriosus include pulmonary arterial hypertrophy, pulmonary hypertension, and weakening of the myocardium in the neonate (Dice and Bhatia 2007). In contrast, early closure of the ductus arteriosus in utero as a result of indomethacin treatment may lead to pulmonary hypertension, fetal hydrops, and intrauterine death (Aker, et al.). Thus, prolonged prostaglandin inhibition has too many severe adverse effects that are not worth the potential relaxative effects.

The β2 adrenergic receptor agonists are less commonly used as tocolytics. The β2 adrenergic receptor agonists are used to relax pulmonary smooth muscle to treat patients with bronchial asthma and chronic obstructive pulmonary disease (Cazzola et al. 2013). These receptors use G_s pathways to relax smooth muscle. Commonly, the G_s pathway is known to mediate relaxation in smooth muscle via activation of adenylyl cyclase, cyclic AMP accumulation, and protein kinase A activation, but, clinically, in the case of the myometrium, cAMP does not mediate meaningful relaxation (Lai, Tribe, and Johnson 2016). β2 adrenergic receptor agonists have delayed birth up to 48 hours as well, but they do not decrease the rate of preterm birth or the negative outcomes associated with it (Neilson, West, and Dowswell 2014). Additionally, the β2 adrenergic receptor is not even the predominant adrenergic subtype in the myometrium (Schena and Caplan 2019). In order to completely analyze the potential of β adrenergic receptors as tocolytic strategies, we must evaluate and understand the predominant subtype in the myometrium: β3 adrenergic receptor.

β3 Adrenergic Receptor

The β3 adrenergic receptor (β3AR) has been identified as a potential target for developing novel tocolytic strategies for PTL (Asif et al. 2022). At first, when studying the β adrenergic receptors in the smooth muscle of the bladder, researchers discovered that there was a subcategory of the receptors that did not fit the criteria for either the β1 or β2 subtypes (Nergårdh et al. 1977; Schena and Caplan 2019). Afterwards, β3AR was first cloned in 1989 (Emorine et al. 1989) and then universally accepted as a subtype of the β adrenergic receptor family. The β3AR was then found in several different species including mice, rats, goats, dogs, sheep, bovines, and humans (Schena and Caplan 2019; Sasaki et al. 1998; Granneman et al. 1991; Nahmias et al. 1991). The β3AR gene in mice is 81% homologous to human, with the receptor's transmembrane domain 94% homologous (Granneman et al. 1991).

The molecular and physical structure of the β3AR depicts the uniqueness of this receptor compared to its β1 or β2 counterparts. The gene for the β3AR is located on chromosome 8 and is composed of two exons and one intron (Granneman, Lahners, and Chaudhry 1993; Schena and Caplan 2019). Exon 1 of the β3AR gene composes most of the coding region, while exon 2 has only 19 base pairs of coding region. In humans, there are no known splicing variants of the receptor (Van Spronsen et al. 1993; Schena and Caplan 2019). The β3AR contains 408 amino acids forming a seven transmembrane domain (three intracellular loops and three extracellular loops) protein part that belongs to the same G-protein-

coupled receptor family as β1 and β2 adrenergic receptors (Schena and Caplan 2019). The transmembrane domains TM3, TM4, TM5, and TM6 function to support ligand binding, while TM2 and TM7 are involved in the activation of the G protein subunits (Strosberg and Pietri-Rouxel 1996; Schena and Caplan 2019). However, the β3AR has the ability to activate either a G_s or G_i pathway, which gives it the ability to activate a variety of downstream signaling responses (Hadi et al. 2013; Sato et al. 2005). Furthermore, the β3AR does not have phosphorylation sites for protein kinase A (PKA) and β-adrenoreceptor kinase (βARK). Overactivation of the β2 adrenergic receptor promotes βARK to phosphorylate it in order to recruit β-arrestin, which leads to the internalization and desensitization of the β2 adrenergic receptors (Schena and Caplan 2019; Asif, Barnett, and Buxton 2022). As the β3AR lacks the sequences for this phosphorylation, it does not become desensitized after long periods of activation, making it an optimal target for treatment strategies involving chronic exposure.

In humans, the β3AR was identified to be in various organ systems with varying downstream effects. β3AR was found in both white and brown adipose tissue (De Matteis et al. 2002). β3AR is mostly active in the white adipose tissue, activating lipolysis and thermoregulation (Schena and Caplan 2019). In humans, β3AR was found to have lowered expression in obese patients. In mice, β3AR was found to protect against hyperinsulinemia, decrease free fatty acids, protect against severe weight gain, and increase resting energy expenditure (Clookey et al. 2019; Decara et al. 2018; Schena and Caplan 2019). Since β3AR can activate either a G_s or G_i downstream signaling pathway, it can use either a cAMP-PKA or

ERK/MAP kinases to recruit hormone-sensitive lipase or perilipin to begin the process of lipolysis (Robidoux et al. 2006; Schena and Caplan 2019). The ability to use multiple downstream signaling pathways may further support its potential as a tocolytic strategy.

β3AR has been identified in the myocardium in both the atria and the ventricles (Chamberlain et al. 1999). In the ventricles, stimulation of β3AR had a negative inotropic effect on cardiac contractility. The negative inotropic effect was inhibited when preincubated with pertussis toxin, showing that this effect occurs through a G_i mediated pathway (Gauthier et al. 1996; Schena and Caplan 2019). Inhibition of nitric oxide synthase (NOS) prior to β3AR stimulation was shown to decrease the negative inotropic effect of β3AR (Schena and Caplan 2019). This confirms the likelihood of β3AR stimulation increasing nitric oxide (NO) production. The increase in NO production from β3AR stimulation has been seen associated with smooth muscle in other parts of the cardiovascular system as well (Dessy et al. 2004). Preincubation with a soluble guanylyl cyclase inhibitor decreased the effects of β3AR in the myocardium, showing that nitric oxide production may follow the NO-cGMP-PKG pathway (Angelone et al. 2008). Endothelial NOS (eNOS) is mostly involved in the relaxation effects occurring from β3AR in the myocardium; however, neuronal NOS may play a role as well (Gauthier et al. 1998; Schena and Caplan 2019; Watts et al. 2013). The increase in endogenous NO through β3AR activation may further support its use to relax the myometrium.

Contradictory to the effects seen in the ventricle, β3AR activation was shown to have a positive inotropic effect in the myocardium of the atria. This

positive inotropic effect occurred through the stimulation of L-type Ca^{2+} channel. These effects were shown to decrease when β3AR stimulation followed a preincubation of a selective PKA inhibitor, confirming a cAMP/PKA pathway (Skeberdis et al. 2008; Schena and Caplan 2019). The contradictory effects seen in the myocardium have been associated with using β3AR agonists which also have high affinity for binding the other β adrenergic receptors.

In the urinary system, β3AR has been detected in the bladder, prostate, urethra, and kidneys (Schena and Caplan 2019); however, it has been mainly studied in the bladder for being the predominant β adrenergic receptor subtype in the detrusor muscle (Yamaguchi 2002). Stimulation of β3AR relaxes the detrusor muscle in the bladder. This relaxation is thought to occur due to the cAMP/PKA pathway from the G_s pathway because the effects of β3AR stimulation can be inhibited by SQ22536, an adenylate cyclase inhibitor (Uchida et al. 2005). Recent studies have shown that the relaxation of the smooth muscle through β3AR activation occurs through a hyperpolarizing effect from the activation of large conductance Ca^{2+} activated K^+ channel (BK_{Ca}), and this, in combination with the cAMP/PKA pathway, promotes a quiescent state for the detrusor muscle (Frazier et al. 2005; Schena and Caplan 2019).

In the brain, β3AR has been detected in the frontal cortex and hippocampus (Rodriguez et al. 1995; Schena and Caplan 2019). Studies have shown that the β adrenergic receptors affect depressive states (Ressler and Nemeroff 2000; Schena and Caplan 2019). Rat studies have provided evidence that β3AR agonist treatment increases tryptophan, 5-hydroxytryptamine, and norepinephrine release,

and helps to combat depressive effects through serotoninergic activity (Tamburella et al. 2010; Schena and Caplan 2019; Claustre et al. 2008). Since β3AR is found in the hippocampus, it has been associated with memory consolidation (Jhaveri et al. 2010). β3AR was found to modulate glucose metabolism in the brain and neuronal excitability/firing to promote an increase memory retention (Schena and Caplan 2019).

In recent years, identification of β3AR in the human myometrium have lead researchers to describe it as a potential method for mediating relaxation (Rouget et al. 2005a). Although some pathways have been described, such as antioxidant effects in macrophages that may contribute to myometrial quiescence (Hadi et al. 2017), the mechanism underlying β3AR mediated relaxation in the myometrium are incompletely understood. Thus, further insight into the signaling pathways associated with β3AR can help support the idea of pursuing it as a target for tocolytic development.

Currently, there are multiple agonists developed to target β3AR. BRL37344 has recently been most used in studies to elaborate on the role of β3AR in relaxing the detrusor muscle (Schena and Caplan 2019). The pharmacological effects of BRL37344 have been shown to be a bit controversial. Some studies have shown it to have a higher affinity for β3AR over its β1 and β2 counterparts; however, other studies show that it still activates the other β adrenergic receptors (Schena and Caplan 2019). CL316243 is another experimental drug used to test the effects of β3AR activation. Although it is more selective towards the β3AR than other

subtypes and been shown to relax smooth muscle, it has poor potency (Hutchinson et al. 2006; Schena and Caplan 2019).

There have been many β3AR agonists that have been tested in clinical trials. Amibegron (SR58611A) was tested in clinical trials for its antidepressant effects, but was discontinued in Phase III for its lack of efficacy in patients (Claustre et al. 2008; Schena and Caplan 2019). Ritobegron (KUC-7483) was developed to treat overactive bladder syndrome, but was also discontinued in Phase III of clinical trials due to lack of efficacy (Schena and Caplan 2019). Solabegron (GW427353) is currently in Phase II of clinical trials for overactive bladder syndrome (Ohlstein, Von Keitz, and Michel 2012). Vibegron has been approved in Japan for the treatment of overactive bladder syndrome, but it is not FDA approved (Keam 2018). Mirabegron (YM178) is currently the only FDA-approved β3AR agonist for overactive bladder treatment (Takasu et al. 2007). This makes mirabegron an optimal choice for studying the effects of β3AR stimulation and developing novel treatment strategies for different clinical scenarios, such as PTL.

Conclusion

Current tocolytic strategies are ineffective in treating PTL, or preventing the outcomes associated with it. To develop the most efficacious tocolytic strategy, one should focus on utilizing pathways that stimulate multiple factors favoring relaxation. This research will discuss the potential of using a selective β3AR agonist, mirabegron, as a tocolytic strategy. We will determine the signal

transduction pathways stimulated by the β3AR, and determine if other receptors have similar downstream effects. Overall, this research aims to supplement our knowledge about the physiological capabilities of the myometrium, and how it can be used to treat PTL.

Chapter 2

β3 Adrenergic Receptor Signaling in the Human Myometrium

Disclosure Statement

The authors declare they have no conflict of interest. Permission granted to use published work for dissertation (Asif et al. 2022). (Appendix A)

Abstract

Preterm labor leading to preterm birth is the leading cause of infant morbidity and mortality. Although β2 adrenergic agonists fail to provide adequate tocolysis, the expression of the β3 adrenergic receptor in myometrium and its unique signaling suggest a role for β3 agonist in the management of preterm labor. Western blot analysis showed that the β3 adrenergic receptor expression increased in human pregnancy myometrium compared to nonpregnant tissues ($p < 0.0001$). There was no difference in β3 adrenergic receptor expression throughout pregnancy ($p > 0.05$). The addition of the β3 agonist mirabegron in the tissue bath relaxed oxytocin contracted myometrium with an EC_{50} of 41.5 μM. Relaxation was partially blocked by the addition of the eNOS blocker Nω-nitro-L-arginine, or the large conductance potassium channel blocker paxilline. Combination of Nω-nitro-L-arginine and paxilline prevented mirabegron-mediated relaxation. Imaging revealed that the β3 adrenergic receptors are expressed by both myocyte and microvascular endothelial cells isolated from human myometrium. Nitric oxide production measured by 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate revealed that mirabegron stimulated nitric oxide production in myometrial endothelial cells. These data suggest that both endothelial and smooth muscle cells contribute to relaxation through disparate signaling pathways. Repurposing of approved medications tested in human myometrium as uterine tocolytics can advance prevention of preterm birth. These data argue that further examination of β3 adrenergic receptor signaling in myometrium may reveal mirabegron as a useful tocolytic in combination tocolysis regimens.

Introduction

Approximately 15 million preterm births (PTB) before 37 completed weeks of gestation occur annually worldwide (Blencowe et al. 2012). The regulation of birth timing is unknown (Romero et al. 2014). The earliest preterm infants are at risk for major disability (Ray and Lorch 2013; Suman and Luther 2021). In 2018, 10-14% of births in the U.S. were premature depending on maternal ethnicity (Martin, Hamilton, and Osterman 2019). African Americans are disproportionally affected (Rawlings, Rawlings, and Read 1995; McKinney et al. 2017); a recognized health disparity (Culhane and Goldenberg 2011; Manuck 2017), worsened by the CoV-2 pandemic (Thebault et al. 2020). Indeed, although many cases of PTB are unrelated to any known pathology of mother or fetus, recent examination of women who contract COVID-19 while pregnant face a higher risk of delivering very early (Karasek et al. 2021). The risk of very PTB, which occurs at less than 32 completed weeks of gestation, is 60 percent higher for women infected with COVID-19 at some point in their pregnancy, while the risk of giving birth at less than 37 weeks is 40 percent higher in women with CoV-2 infection. For those pregnant women who have underlying health conditions such as hypertension, diabetes and/or obesity as well as COVID-19 infection, the risk of preterm birth rises 160 percent. The continuing emergence of CoV-2 variants underscores the urgency of developing effective tocolytics.

PTB is a major medical issue. Complications for preterm infants such as blindness, respiratory distress syndrome, jaundice, infections, and brain

hemorrhaging (Suman and Luther 2021) are inversely correlated with gestational length. Long-term issues may arise as well, such as chronic lung disease and neurodevelopmental disabilities (Luu et al. 2017). There are several risk factors linked to PTL, including: infections such as chorioamnionitis, smoking or the use of illicit drugs, advanced maternal age (>35 years old), ethnicity of the mother, polyhydramnios, and chronic maternal conditions, such as high blood pressure or diabetes (Purisch and Gyamfi-Bannerman 2017). Spontaneous PTL (sPTL) accounts for half of PTL cases without known cause (Deressa et al. 2018). Treatments for PTL are ineffective (Reinebrant et al. 2015; Crowther et al. 2014; Flenady et al. 2014; Neilson et al. 2014) no matter the underlying cause. Developing new tocolytic strategies requires that we understand the myometrial dysfunction(s) that result in preterm labor leading to preterm birth.

The importance of β3 adrenergic receptorss in the production of quiescent mediators (Dessy et al. 2004), combined with its upregulation in the myometrium during pregnancy, make it a prime target for tocolytic strategies (Rouget et al. 2005b). The β3 adrenergic receptor (β3AR) was first identified in 1989 (Emorine et al. 1989) and then universally accepted as a subtype of the βeta adrenergic receptor family. The β3AR is present in the small intestine, adipose tissue, vascular endothelium, and the smooth muscle of the colon and bladder (Deeks 2018). The β3AR has also been described in the myometrium and shown to mediate relaxation (Rouget et al. 2005a), where it was determined that the β3AR agonist reduced myometrial contractions in monkeys and confirmed the failure of β2AR stimulation by salbutamol to do so. The β3AR subtype differs from β1 and β2 receptors in the

third intra-cytoplasmic loop and C-terminal tail, where the β3AR lacks the consensus sequence for phosphorylation by βAR kinase (βARK). βARK mediates β-arrestin recruitment, followed by internalization and homologous β2AR desensitization. Without a consensus sequence for βARK, the β3AR is not readily downregulated (Schena and Caplan 2019). As such, the β3AR response has longer duration of action and is thus better suited as a target for tocolytic strategy (Rouget et al. 2004). While these data are encouraging, they do not explain the mechanisms underlying β3AR stimulated relaxation needed to explore novel tocolytic development.

Multiple underlying downstream mechanisms in the myometrium have been associated with the β3AR, including either or both G_i and G_s pathways, each with unique downstream effects (Hutchinson et al. 2002; Hadi et al. 2013). Commonly, the G_s pathway is thought to mediate relaxation in smooth muscle *via* cyclic AMP accumulation and protein kinase A activation. In the case of the myometrium it may not (Lai, Tribe, and Johnson 2016). Previous work from Croci *et al.* imagined the therapeutic potential of β3AR stimulation, but did not explore signaling beyond assumptions that cAMP sub-served relaxation (Croci et al. 2007). One pathway that has been described is the β3AR antioxidant effects in macrophages of the myometrium (Hadi et al. 2017). Another pathway involves coupling of the β3AR to BK_{Ca}, large conductance calcium-activated potassium channel (Doheny et al. 2005). BK_{Ca} is the most prominent potassium channel in the myometrium, thought to play playing a major role in regulating cell membrane potential and myometrial quiescence (Anwer et al. 1993). The mechanisms underlying β3AR mediated

relaxation in the myometrium are incompletely understood and may be a result of a combination of different pathways involving other targets, such as nitric oxide (NO) generation.

β3AR has been associated with the activation of NO synthase, which in turn generates NO, resulting in vascular smooth muscle relaxation (Dessy et al. 2004). L-Arginine is transported into endothelial cells where it interacts with endothelial NOS (eNOS) to produce NO (Palmer et al. 1988). NO activates soluble guanylyl cyclase in the underlying muscle to produce cyclic GMP, which then activates protein kinase G, that phosphorylates proteins including myosin phosphatase to relax vascular smooth muscle (Francis et al. 2010). In the myometrium, NO has been shown to relax the tissue in a cGMP-independent manner (Kuenzli et al. 1996; Kuenzli et al. 1998; Bradley et al. 1998; Barnett et al. 2020; Barnett et al. 2018). Regardless of the NO mediated relaxation pathway, the role of eNOS in the β3AR response in myometrium will be addressed here because it may be that existing drugs such as the β3AR agonist mirabegron (Myrbetriq™) could be proposed as part of a combination tocolytic.

Biochemical pathways exploited for tocolysis are inadequate (Siricilla et al. 2020). The unique characterization of the β3AR and its relaxation of myometrium render it a viable target for novel tocolytic strategies. The mechanisms behind this effect are yet to be fully comprehended. This study will investigate the potential of mirabegron, a β3AR selective agonist, as a potential tocolytic, and determine the cellular mechanisms underlying β3AR mediated relaxation.

MATERIALS AND METHODS

Tissue collection: Pregnant human myometrium tissue collection from singleton pregnancies was carried out under informed consent. We adhered to an inclusion/exclusion and stratification paradigm to allow our results to be harmonized with those of other labs (Myatt et al. 2012). Within 20 min of removal, tissues are transported by us to the lab in cold Krebs buffer containing, in mM, NaCl (118), KCl (4.75), $CaCl_2$ (2.5), KH_2PO_4 (1.2), $NaHCO_3$ (25), $MgCl_2$ (1.2), dextrose (20), and is adjusted to pH 7.4. Tissues were dissected microscopically in thin strips (0.8l x 0.3w x 0.2d cm), devoid of obvious blood vessels, and used for primary cell cultures, contractile studies, or a portion stored at -150^{o}C. We collected non-Hispanic white, Hispanic, and Native American (Table I). We collect all de-identified data on the treatments and pregnancy course prior to surgery as well as the presence and degree, or absence of infection, mother's BMI, sex of the infant, smoking status of the mother and social indicators including socioeconomic status, prenatal care, and maternal education when available. Patient identifiers are not collected; case numbers are used for reference. Exclusion criteria include age <18 years, any history of drug abuse, long-term use of tocolytics >48 hours (to control confounding factors), co-morbid diagnoses such as chorioamnionitis, HIV infection or AIDS, hepatitis C infection, rupture of membrane (PROM/PPROM), uncontrolled diabetes, renal disease, preeclampsia, intrauterine growth restriction (IUGR), and any use of steroids other than dexamethasone/ betamethasone.

Myometrial Tissue State	Age (Years)	White	Hispanic	Native American	Diagnosis
Hysterectomy	26-48	12	2	0	Menorrhagia (6), Dysmenorrhea (3), Uterine Fibroids (2), Abnormal Uterine Bleeding (1), Omental Mass (1), Endometriosis (1)
Term Pregnant (37-39 weeks)	20-40	20	3	0	Repeat C/S (15), Breech (5), Fetal Macrosomia (2), Low Fetal HR (1)
Term in Labor (37-39 weeks)	22-42	13	1	0	Repeat C/S (5), Fetal Distress (3), Failure to Descend (2), Low Fetal HR (1), Uterine Fibroids (1), Fetal Macrosomia (1), Thin Uterine Lining (1)
Preterm (28-34 weeks)	21-37	4	1	1	Preeclampsia (2), Placenta Previa (2), PROM (1), CIN (1)
Preterm in Labor (27-36 weeks)	20-40	3	3	0	Breech (2), Placenta Previa (1), Repeat C/S (1), Placenta Accreta (1), Fetal Position (1)

Table 1. Patient Demographics

Table 1: Patient Demographics- The table depicts the different demographic of women who had a Caesarean section and donated a piece of tissue from their myometrium. Term pregnant tissue had the largest number of collections, and a majority of these came from women undergoing a repeat C/S. Preterm laboring tissue had the lowest number of collections due to the rareness of a preterm women undergoing C/S while in labor. A majority of the demographic were White, with very few Hispanic and Native American.

Contractile studies: Dissected strips of myometrium are hung in three 4-channel horizontal tissue bath systems (Danish Myo Technology 820MS, Hinnerup, Denmark), while they are submerged in oxygenated (95% O_2 and 5% CO_2) Krebs buffer at 37^{o}C. The strips are attached to transducers in each bath and pulled to 2 gms tension. KCl (60mM) was applied for 3 min to stimulate contractions, followed by a washout and a one-hour equilibration period. After spontaneous contractions were established, myometrial strips were treated with 8nM oxytocin to achieve maximal contractions. Transducer voltages were converted to digital signals and transferred to the LabChart software (ADInstruments) for analysis (Barnett et al. 2020). Tissues were treated with mirabegron (MBG) (Tocris, Bristol, United Kingdom) for one-hour, or with a combination of Nω-Nitro-L-arginine (L-NNA) (Sigma-Aldrich, St. Louis, U.S.A.), SR59230A (Tocris, Bristol, United Kingdom.), or Paxilline (Sigma-Aldrich, St. Louis, U.S.A.) 15 min prior to MBG. Larger doses of MBG were not used to avoid a 1% concentration of DMSO in organ baths. Washouts were performed after each dosing period to observe a return in contractions. Tissues that did not respond to the KCl-challenge or did not display any post wash out contractions at the end were not included.

Western blot: Tissue samples were crushed using a Mortar and Pestle precooled in LN_2. Crushed tissue samples were added to MAPK buffer containing in mM, (Tris-HCL pH 6.8 (60), glycerol (1%), SDS (2%), leupeptin (0.001), EGTA (1) EDTA (1), AEBSF (1), Na_3VO_4 (1), and protease/phosphatase inhibitors (Sigma

Aldrich, St. Louis, U.S.A.) followed by 5 min in a tissue homogenizer on ice. For cell protein lysates, cells are trypsinization for 5 min, centrifuged for 10 min (650 RCF) and the cell pellet resuspended in MAPK buffer with sonication on ice for 3 min. Protein quantification on both tissue and cell protein lysates were performed via EZQ (Thermo Fisher, Waltham, U.S.A.). For both the β3AR and eNOS protein lysate, 40μg of protein was loaded into a 4-15% Mini-Protean TGX precast gel in standard tris/glycine PAGE buffers (BioRad, Hercules, U.S.A.), and proteins separated at 200V, and transferred to nitrocellulose blocked in Licor blocking buffer for 1 hr. Blots were labeled with either primary mouse anti-β3AR (1:1000, sc-515763, Santa Cruz Biotechnology, Dallas, U.S.A.) or primary mouse anti-eNOS (1:1000, ab76198, Abcam, Cambridge, United Kingdom), and then labeled with secondary Alexa-Fluor 680 donkey anti-mouse (1:25000, ab175774, Abcam, Cambridge, United Kingdom). Both beta-3 adrenergic receptor and eNOS were normalized to their respective GAPDH concentrations in each sample using the primary rabbit anti-GAPDH (1:1000, 2118S, Cell Signaling Technology, Danvers, U.S.A.) and the secondary IRDye 800 donkey anti-rabbit (1:25000, 926-32213, Licor Biotechnology, Lincoln, U.S.A.).

Cell culture: Enzyme solution was prepared containing 20mg collagenase, 10mg Trypsin (27250-018, Gibco, Waltham, U.S.A.), and 10 ml of solution containing 5 ml of MACS buffer and 5 ml of Gibco Dulbecco's Modified Eagle Medium (DMEM, 11995-065, Gibco, Waltham, U.S.A.). The resulting solution incubated at 37^{o}C with small, dissected pieces of myometrium in three successive agitation steps (90 sec) followed by two 45-min incubation periods at 37^{o}C with

rotation. The digestion is triturated x 3 and filtered through a 100-micron sterile mesh. Cells derived in this fashion are grown to 80% confluency and then preincubated with FcR blocking reagent, then separated over CD31+ bead LS columns using a MidiMACS separator (Miltenyi BioTec, Bergisch Gladbach, Germany) into CD31+ pregnant human myometrial endothelial cells (phMEC) and CD31-pregnant human uterine smooth muscle cells (phUSMC). Once 80% confluent in P0 culture, cell culture is grown to P3 and employed or frozen for future experiments at $1x10^6$ cell/ml. Telomerized human uterine smooth muscle cells (hTRT, Heyman et al. 2013) were grown between P20-P30. The phMEC were grown in endothelial basal medium 2 (PromoCell, St. Louis, U.S.A.) containing 10% FBS and 1% penicillin, while the phUSMC and hTRT cells were grown in Gibco DMEM (Gibco, Waltham, U.S.A.) containing 10% FBS and 1% penicillin.

Immunofluorescence: Cells from CD31+ bead separation were plated on 35mm glass bottom dishes (MatTek Corporation, Ashland, U.S.A.) and grown to 70% confluence. Cells are incubated for 15 min with 4% paraformaldehyde, followed by 0.5% triton-x for 5 min and 5% BSA blocking buffer for 1 hr with three PBS washes between steps. Cells were then stained with an anti-β3AR antibody (1:1000, sc-515763, Santa Cruz Biotechnology, Dallas, U.S.A.) or anti-CD31 antibody (ab949, Abcam, Cambridge, United Kingdom). Following primary staining, cells were stained with secondary antibody (Alexa Fluor 594 ab150080, Abcam, Cambridge, United Kingdom) and imaged under confocal microscopy (10X magnification). In addition to the primary proteins of interest, cells were also stained with wheat germ agglutinin (WGA) conjugated to Alexa Fluor 488

(W11261, Thermo Fisher Scientific, Waltham, U.S.A.) to establish cell boundaries, followed by DAPI mounting medium (H-1500, Vector Laboratories) to identify the nucleus. All images were taken with negative controls (lack of experimental condition primary antibody) to verify the absence of nonselective binding (data not shown).

DAF-FM diacetate assay: Selected phMEC and phUSMC, along with hTRT cells, were passed onto a half area, 96 well microplate (675076, Greiner Bio One, Kremsmünster, Austria) at 4,000 cells/well. Cells are grown to 80% confluence. The phMEC were washed x-three with phenol red free EGM-2 media (C-22216, PromoCell, St. Louis, U.S.A.), while the phUSMC and hTRT cells were washed with phenol red free DMEM (21063029, Thermo Fisher, Waltham, U.S.A.). Cells were then incubated with DAF-FM (100μM) (Ex/Em 495/515, D23844, Thermo Fisher, Waltham, U.S.A.) for 30 min at 37^{o}C, washed and fresh phenol red free medium added and incubated for an additional 30 min at 37^{o}C to allow for complete de-esterfication of intracellular diacetates. phUSMC and phMEC were treated with MBG (100μM) or left alone as control cells, and then processed on the VICTOR Nivo Multimode Microplate Reader (Perkin Elmer, Waltham, U.S.A.) for 1 hr at 37^{o}C. Only wells with live cells within 2 standard deviations from the mean for each condition were used for statistical analysis. hTRT cells were treated with S-Nitrosoglutathione (300μM) (GSNO, A.G. Scientific, San Diego, U.S.A.) as positive controls to verify the DAF-FM diacetate assay (data not shown).

Statistical analysis: Experiments involving contractile studies employed 3-5 patients for each data set. Replicate variation was controlled by multiple tissue

strips from each patient. Contractile data is presented as area under the curve (AUC) by calculating the integral relative to the minimum baseline of the respective contraction. The last three contractions in the last 15 min for each dosing period was analyzed. Each tissue strip was double normalized, first to itself prior to dosing, and then to a control tissue strip at the respective data collection time point. Control tissue strips were dosed with volume equivalents of the drug (DMSO for MBG, SR59230A, paxilline). The average of the AUC values from the control tissue strips were set to 100% so that data from experimental strips would show percentage relative to baseline. In the western blot experiments, each sample was from a different patient, representing its own 'n.' Statistical analysis for contractile studies and western blots were completed using two-tailed, unpaired t-tests. In the DAF-FM diacetate assay, each individual well containing cells was considered its own 'n.' Both experimental and control conditions for the cells had results from a baseline of wells filled with DAF-FM and media alone subtracted from the conditions. Afterwards, the untreated conditions were subtracted from the treated conditions for each cell type. Then a 0 to 1 normalization was performed for the average of the phUSMC, and the cells were normalized against it to measure relative fold change in fluorescence. Each individual time point was compared vertically to its corresponding time point in their cell type, and a two-tailed, unpaired t-test was performed to detect significance. Data points within each cell type were compared to each other using a one-way ANOVA to detect significance within their respective group. All statistical analysis was done using Prism (v. 8.4.3, Graphpad Software, San Diego, U.S.A.) with significance set to $p<0.05$.

RESULTS

β3AR and eNOS Expression in Disparate States of Pregnancy

Gene expression has been shown to vary widely in the myometrium of women who experience preterm labor, thus amplifying the need for understanding the expression of target proteins, such as β3AR and eNOS, to determine if their expression indicates a vital role in mediating labor (Paquette et al. 2018; Enquobahrie et al. 2009; Knijnenburg et al. 2019; Barnett et al. 2020). β2AR expression has been shown to decrease in the human myometrium after stimulation with βeta mimetics (Berg et al. 1985); however, the β3AR does not desensitize like the β2AR, and the β3AR is the dominant βeta adrenergic receptor in the human myometrium (Rouget et al. 2004; 2005b). We examined myometrial β3AR expression in disparate states of pregnancy for the first time. Western blot was performed using protein lysates from human myometrial tissue samples nonpregnant (NP) (n=13), term nonlabor (TNL) (n=12), term labor (TL) (n=12), preterm nonlabor (PTNL) (n=6), and PTL (n=6), and each sample was normalized to their respective GAPDH concentration (Fig. 1A). Results showed an increase in β3AR concentration during pregnancy when compared to the non-pregnant state ($p<0.0001$). During gestation and labor there was no difference in β3AR expression ($p>0.05$). This increase in β3AR expression occurring as a result of pregnancy suggests its importance as a target for promoting quiescence during gestation.

To investigate the role of β3ARs in NO production, western blot was performed to determine the relative amount of eNOS expressed in human

Fig. 1

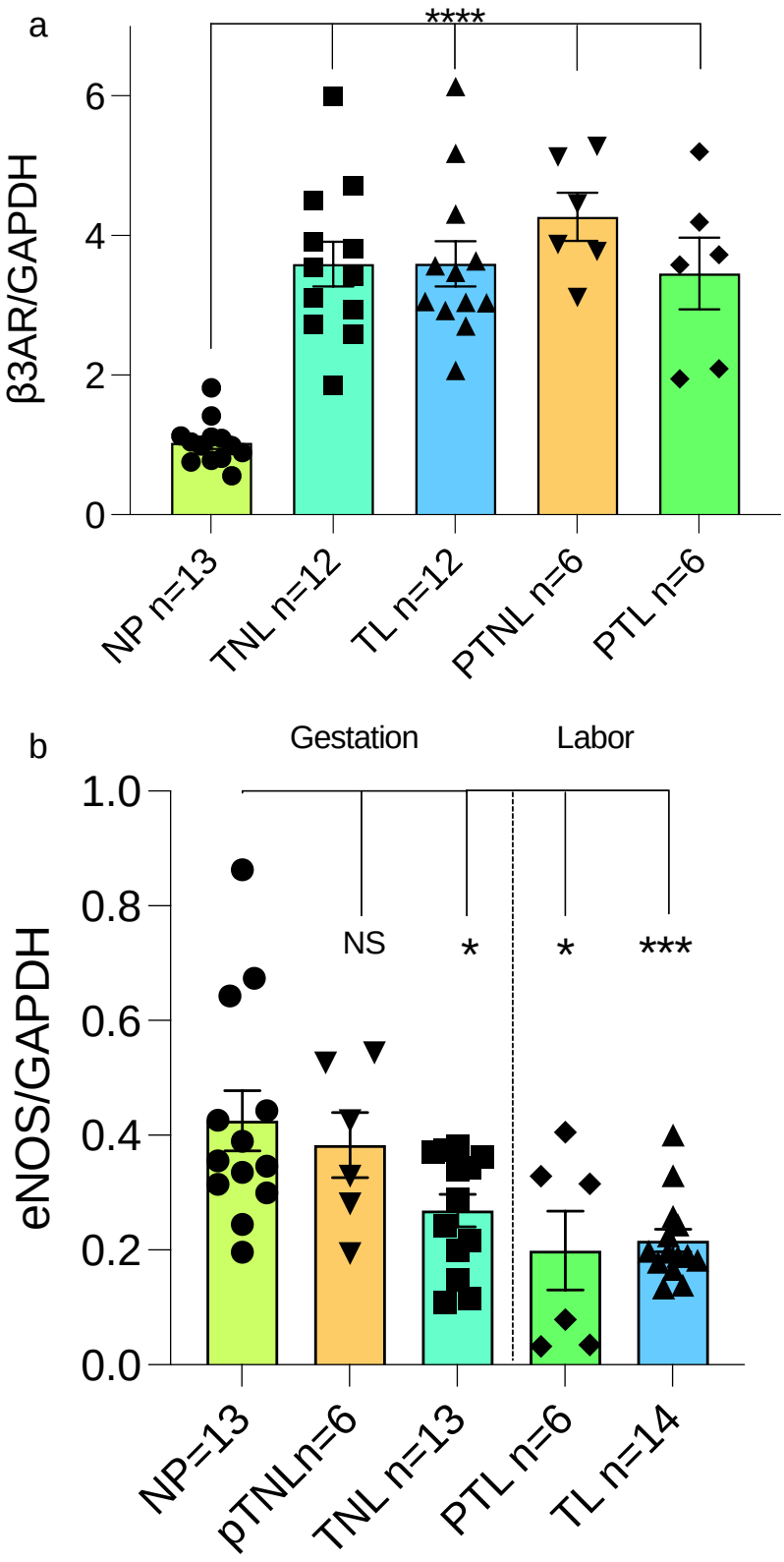

a
****
β3AR/GAPDH
0
2
4
6
NP n=13
TNL n=12
TL n=12
PTNL n=6
PTL n=6
b
Gestation
Labor
eNOS/GAPDH
0.0
0.2
0.4
0.6
0.8
1.0
NS
*
*
***
NP=13
pTNLn=6
TNL n=13
PTL n=6
TL n=14

Figure 1: β3AR and eNOS expression in disparate states of pregnancy A.) The β3AR increases throughout pregnancy in PTNL ($p<0.0001$), PTL ($p<0.0001$), TNL ($p<0.0001$), and TL ($p<0.0001$) when compared to a NP state. There is no difference between the different states of pregnancy ($p>0.05$). B.) eNOS expression decreases as gestation time increases (PTNL $p>0.05$, TNL $P<0.05$), and decreases in the state of labor regardless of gestation time (PTL $P<0.05$, TL $p<0.001$) when compared to a NP state.

myometrial tissue samples from NP (n=13), TNL (n=13), TL (n=14), PTNL (n=6), and PTL tissue samples (n=6) and each sample was normalized to GAPDH (Fig. 1B). Results showed that there was no observed difference in eNOS expression in PTNL when compared to NP samples ($p>0.05$), but decreased as gestation time increased to term ($p<0.05$). Also, there is a decrease in concentration of eNOS in term laboring ($p<0.001$) and preterm laboring ($p<0.05$) samples when compared to NP samples.

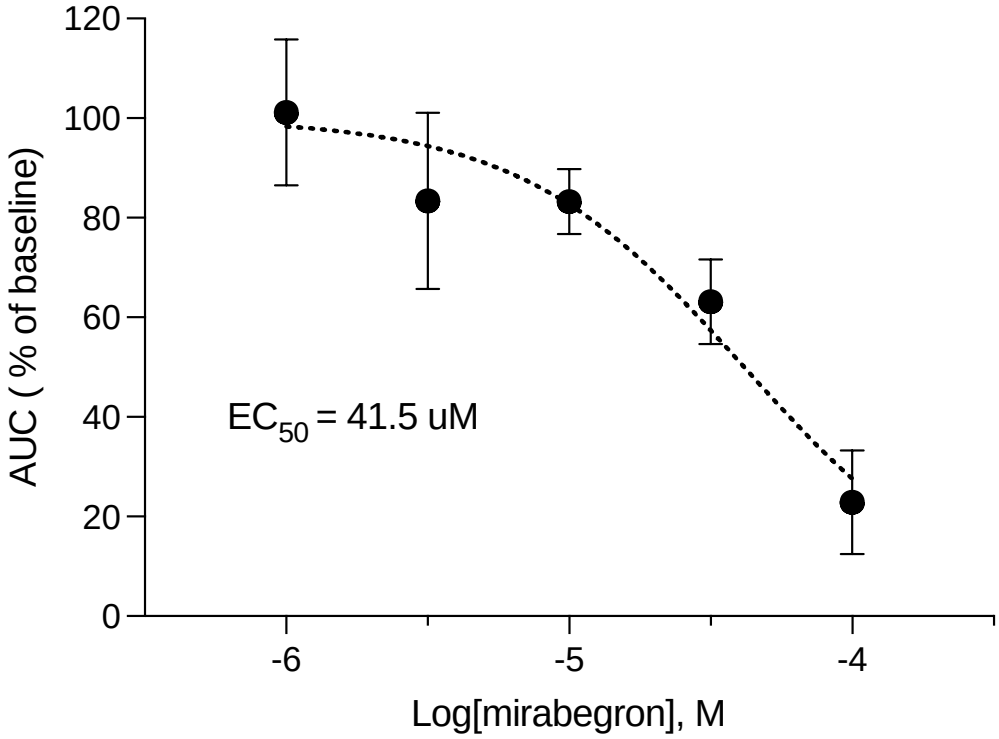
Effect of Mirabegron on Human Myometrial Tissue

MBG is an FDA approved β3AR selective agonist used to treat overactive bladder syndrome. MBG has been shown to relax smooth muscle on contracting bladder strips (Takasu et al. 2007). Thus, we examined the effects of MBG on human myometrial tissue strips in TNL patients. Dose response was performed to determine the EC_{50} for MBG in human myometrium (Fig. 2A). After the myometrial tissue strips reached maximum contractile activity following 8 nM oxytocin, they were treated with MBG in half-log doses ranging from 1 μM to 100 μM, or a volume equivalent of MBG diluent, DMSO (n=5). Analysis using the AUC resulted in a MBG-mediated EC_{50} of 41.5 μM. Traces of contracting uterine smooth muscle treated with 41.5 μM MBG and its volume equivalent buffer control, each compared to their respective baseline, showed a drop in about 50% AUC and a return of contractions following washout (Fig. 2B).

To determine the mechanistic pathways responsible for the observed relaxation of oxytocin contractions, TNL tissue strips were pretreated with either 10 μM SR59230A (a selective β3AR antagonist, n=3), 100μM L-NNA (NOS

Fig. 2

a


120
100
80
60
40
20
0
AUC (% of baseline)
EC50 = 41.5 uM
-6
-5
-4
Log[mirabegron], M

b

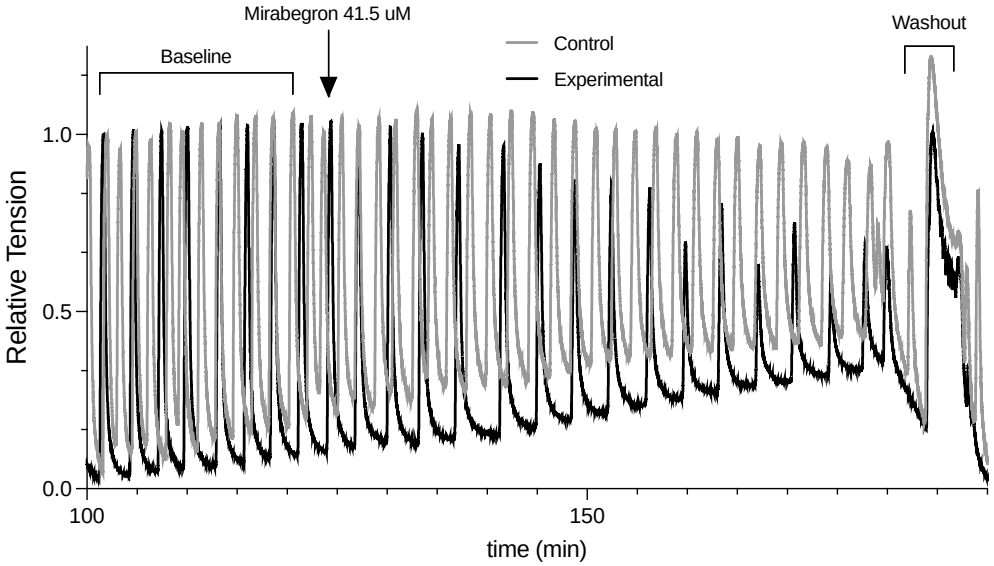

Mirabegron 41.5 uM
Baseline
Control
Experimental
Washout
1.0
0.5
0.0
Relative Tension
100
150
time (min)

Figure 2: EC_{50} of mirabegron on Human Myometrial Tissue A.) Human myometrial tissues (TNL) were challenged with KCl and given oxytocin (8nM) to maximize baseline contractions. Tissues were dosed with mirabegron for 1 hour at half-log doses ranging from 1 μM to 100 μM (n=5). The AUC relative to the percentage of the baseline for each dose was plotted, showing a EC_{50} of 41.5 μM. B.) Depicts a trace of human myometrial tissues (TNL) being dosed with mirabegron (41.5 μM) or volume equivalent DMSO (control) relative to the maximum contractions established in the baseline using oxytocin. Trace depicts an effective EC_{50} along with a return in contractions during the last washout.

inhibitor, n=3), 10μM paxilline (BK_{Ca} inhibitor, n=3), a combination of 100μM L-NNA and 10μM paxilline (n=3), or untreated (8 nM oxytocin alone) for 15 min. Following this pretreatment period, tissues were dosed with 100μM MBG, except for tissues pretreated with SR59230A, which were either given 100μM MBG or left untreated. Control organ baths were dosed with volumetric equivalent DMSO. Results were graphed against the percentage of AUC relative to the baseline for their respective conditions (Fig. 3). Tissues treated with SR59230A alone showed 113% in AUC relative to baseline, SR59230A plus 100μM MBG showed a 115% AUC relative to baseline. MBG (100μM) exhibited an AUC of 18.27% relative to baseline, and 100μM MBG plus L-NNA demonstrated an AUC of 65.83% relative to baseline. Furthermore, 100μM MBG plus paxilline showed an AUC of 63.3% relative to baseline. A combination of MBG, L-NNA, and paxilline had an AUC of 116% relative to baseline. There was no difference when comparing the AUC of SR59230A and SR59230A with MBG ($p>0.05$). Tissue treated with SR59230A plus MBG showed a difference in AUC compared to the combination of MBG and L-NNA ($p<0.05$) and showed a difference in AUC to MBG alone ($P<0.001$). Tissue dosed with a combination of L-NNA and MBG showed a difference in AUC to tissue dosed with only MBG ($p<0.05$). There was a distinction between the AUC in tissue treated with MBG alone and MBG combined with paxilline ($p<0.01$). When tissue strips were co-administered MBG, L-NNA, and paxilline, there was a clear contrast in AUC when compared to MBG alone ($p<0.01$). There was still a difference in AUC between MBG plus paxilline and MBG combined with paxilline and L-NNA

Fig. 3

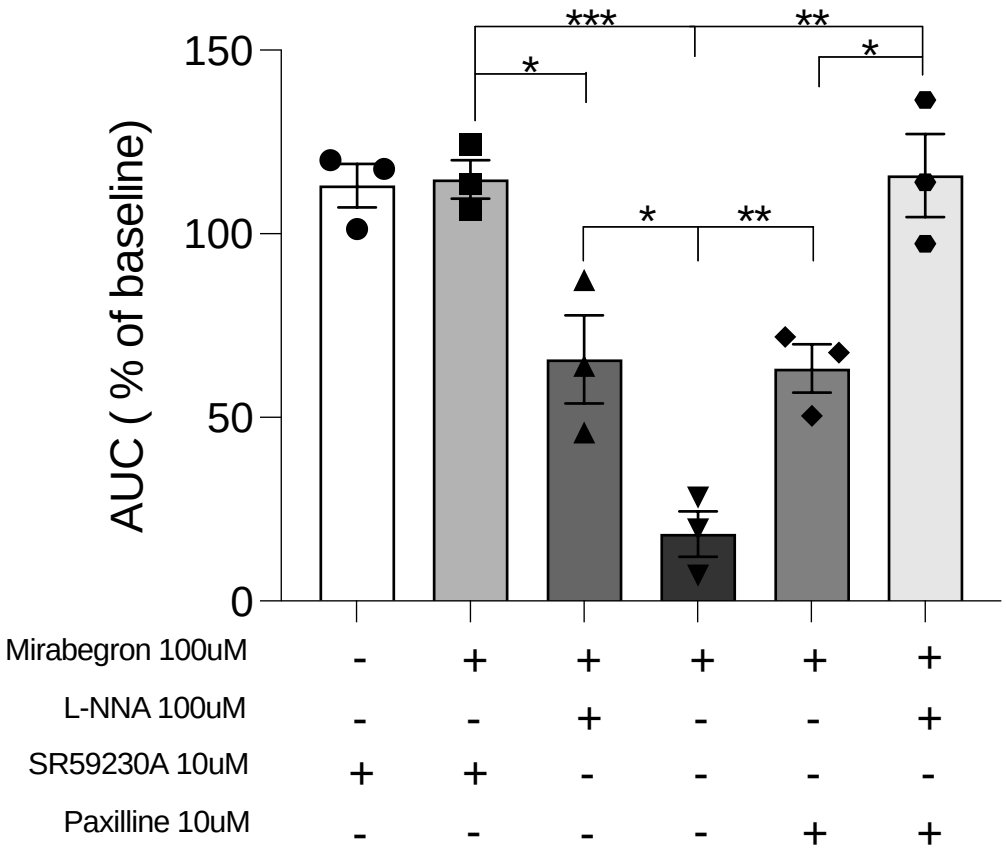

AUC (% of baseline)
150
100
50
0
***
**
*
*
*
**
Mirabegron 100uM
L-NNA 100uM
SR59230A 10uM
Paxilline 10uM
- + + + + +
- - + - - +
+ + - - - -
- - - - + +

Figure 3: Inhibition of mirabegron through different mechanistic pathways – AUC presented as relative to the percentage of baseline. SR59230A ± mirabegron show no difference in AUC ($p>0.05$). Mirabegron had a significantly lower AUC than SR59230A + mirabegron ($p<0.001$), L-NNA + mirabegron ($p<0.05$), paxilline + mirabegron ($p<0.01$), and mirabegron in combination with L-NNA + paxilline ($p<0.01$). There was a distinction in AUC between mirabegron + SR59230A and mirabegron + L-NNA ($p<0.05$). Also, there was a distinction between mirabegron + paxilline and mirabegron in combination with L-NNA + paxilline ($p<0.05$). Mirabegron + SR59230A showed no difference in AUC when compared to mirabegron in combination with L-NNA + paxilline ($p>0.05$).

($p<0.05$). Lastly, there was no significant change in AUC when comparing MBG plus SR59230A and MBG plus paxilline and L-NNA ($p>0.05$).

β3AR Expression in phMEC and phUSMC

Immunofluorescence was performed using primary myometrial cells (TNL, P3) following CD31+ selection to isolate phMECs from phUSMCs. Both phMEC and phUSMC were labeled with DAPI to detect the nucleus, wheat germ agglutinin (WGA) conjugated to Alexa Fluor 488 to visualize the cell membrane, and with primary β3AR and secondary Alexa Fluor 594 antibodies (Fig. 4A). Bright field microscopy demonstrated the expected cellular phenotype for the phMEC and phUSMC in the WGA stain, and it depicted β3AR in both the phMEC and phUSMC compartments. Immunofluorescence was performed to confirm the selection of phMEC versus phUSMC. Cells were stained with DAPI and CD31+ primary and Alexa Fluor 594 secondary antibodies. Images depicted that CD31+ cells were identified in phMEC and not phUSMC (Fig. 4B). Micrograph images were taken showing isolated endothelial (top) and myocyte (bottom) cells from TNL primary culture (Fig. 4C).

After obtaining images of the β3AR in phMEC and phUSMC, we next quantified the expression of the receptor in each cellular compartment. Western blot was performed in cellular lysates of phMEC (n=3) and phUSMC (n=3), with each sample being normalized to their respective GAPDH concentration (Fig. 4D). Results showed a larger β3AR concentration in phUSMC when compared to phMEC ($p<0.05$).

Fig. 4

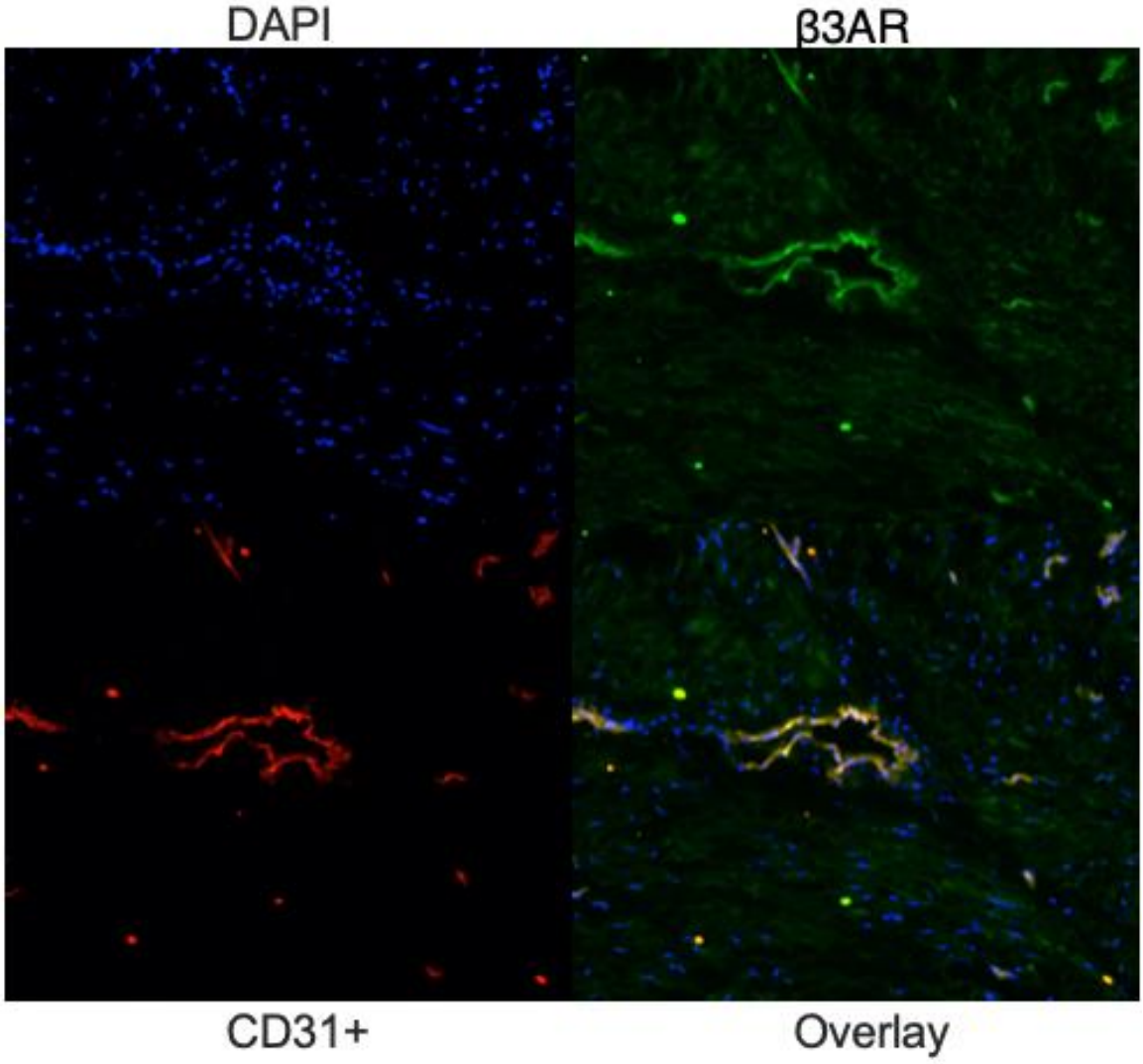


Figure 4: Detection of β3AR and CD31 + in TNL tissue – TNL myometrial tissue was cryosectioned at 15 microns and stained with DAPI, β3AR, and CD31 + antibodies. Immunofluorescence confirms that both β3AR and CD31 + cells are present in the tissue. β3AR is colocalized with CD31 + cells, but is also expressed in the surrounding tissue as well

Fig. 5

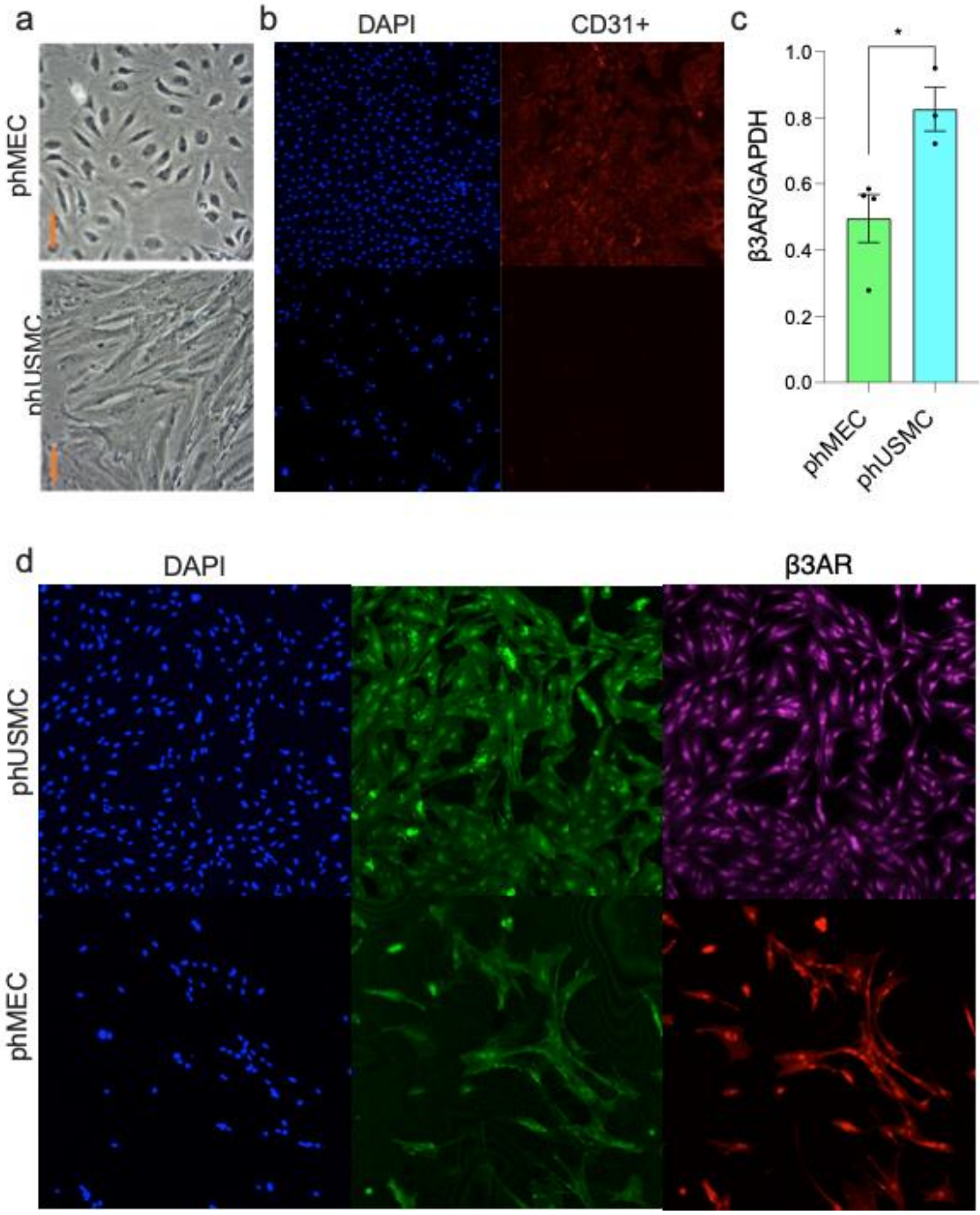

a
b
DAPI
CD31+
c
phMEC
phUSMC
β3AR/GAPDH
1.0
0.8
0.6
0.4
0.2
0.0
*
phMEC
phUSMC
d
DAPI
β3AR
phUSMC
phMEC

Figure 5: Detection and quantification of β3AR in phMEC and phUSMC - Primary cells of the myometrium (TNL) were grown out in tissue culture and selected into phMEC and phUSMC (P3). A.) Micrograph images of phMEC (Top) and phUSMC (bottom) from TNL primary cells on day 3 following selection. Orange bar insert = 100 micron. B.) Both phMEC and phUSMC were stained with DAPI and primary CD31+ antibodies with secondary Alexa Fluor 594 antibodies. phMEC expresses CD31+ cells, while phUSMC shows no expression of CD31+ cells. C.) Western blot confirms quantifiably the expression of β3AR in both phMEC (n=4) and phUSMC (n=3), and depicts a larger β3AR expression in phUSMC versus phMEC ($p<0.05$). D.) phMEC and phUSMC were stained with DAPI, WGA conjugated with Alexa Fluor 488 (cellular membrane), and primary β3AR antibodies with secondary Alexa Fluor 594 antibodies. The WGA images display the phenotype expected from phMEC and phUSMC, and both phMEC and phUSMC express the β3AR.

Nitric Oxide Production in phMEC versus phUSMC through the β3AR

It has been well established that the β3AR is associated with NO production in vascular smooth muscle (Dessy et al. 2004), and that NO can relax the myometrium (Kuenzli et al. 1998; Kuenzli et al. 1996; Barnett et al. 2020). Thus, we next aimed to determine whether the stimulation of the β3AR can increase NO production in the myometrium, and if so, in which cellular compartment.

In order to quantify the amount of NO being produced from β3AR, cells were preincubated with DAF-FM diacetate, which has been shown to detect intracellular NO (Lewis et al. 2016). NO production was quantified in phMEC and phUSMC following β3AR stimulation. All cells were incubated with DAF-FM for 30 min, followed by a 30 min post media change incubation to allow for de-esterfication. Both phMEC and phUSMC were each split into two groups, one group was administered MBG (100μM), while the other was left untreated. Data was recorded at 11 time points ranging from t=0 min to t=60 min in 6-minute intervals. The phUSMC (n=18) and phMEC (n=12) were plotted as a relative fold increase over the untreated control and normalized against the average of the phUSMC (Fig. 5). No significant increase in NO generation was seen in phUSMC, while a significant increase in NO production was seen in phMEC data ($p<0.0001$). There was a significant increase in NO production in the phMEC over the phUSMC at all time points ≥ t=36 min ($p<0.05$).

Fig. 6

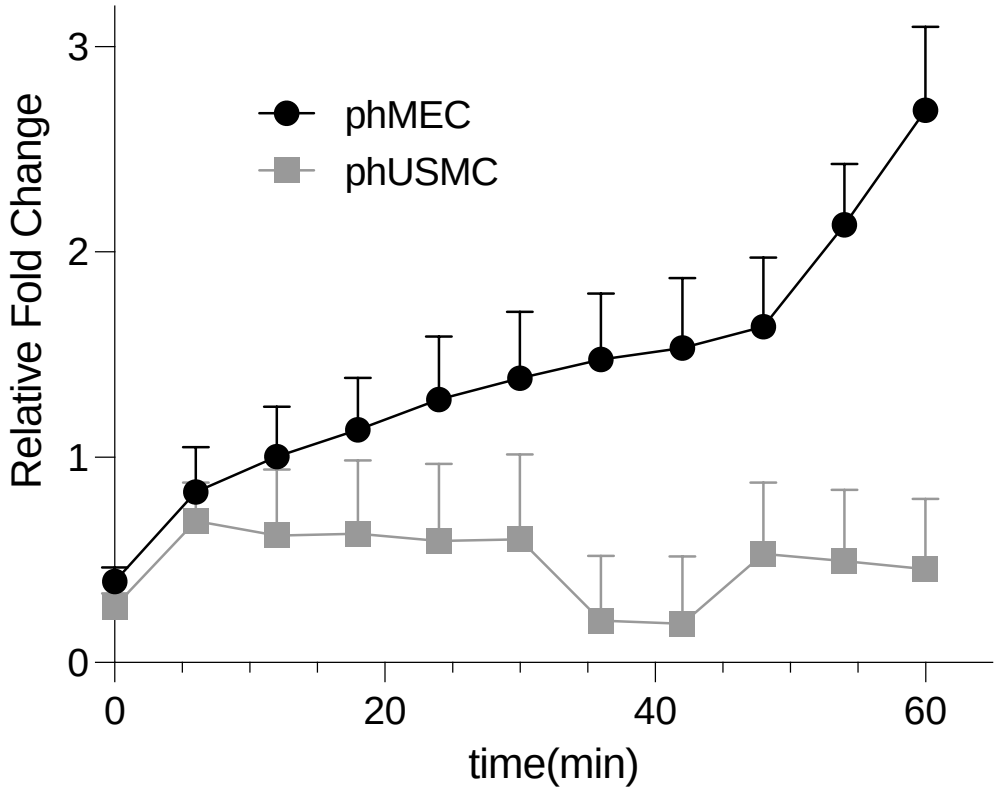


Figure 6: DAF-FM detection in mirabegron dosed phMEC and phUSMC – phMEC (n=12) and phUSMC (n=18) were preincubated with DAF-FM (30 min) then dosed with 100µM mirabegron (±). NO generation (DAF-FM fluorescence 495/530) was measured in 6-minute intervals for 1 hr. Only wells with live cells within 2 standard deviations from the mean for each condition were used for statistical analysis. Data is plotted as a relative fold increase in fluorescence over untreated control. NO generation in phMEC cells increased significantly over phUSMC at all time points ≥ t=36 min ($p<0.05$). One-way ANOVA confirms no difference between the phUSMC data points but a significant difference between the phMEC data points ($p<0.0001$).

Discussion

Preterm labor continues to be the leading cause of morbidity and mortality in neonates (Suman and Luther 2021; Robinson et al. 2001; Norwitz and Robinson 2001). Canonical pathways employed by current tocolytic strategies have proven to be ineffective (Siricilla et al. 2020) at delaying parturition, and it is imperative that novel approaches to tocolytics be developed in order to prevent preterm birth.

Previous studies have shown that the unique characteristics of the β3AR , which include being resistant to the βAR kinase/arrestin downregulation pathway and being abundant in term myometrium (Rouget et al. 2004; 2005b), make it a potential target for tocolytic approaches. We show that β3AR expression increased in disparate states of pregnancy (Fig. 1). This increase in the β3AR supports the importance of the role it may play in maintaining quiescence throughout gestation and its potential as a target for tocolytic strategy.

The potential of the β3AR as a tocolytic led us investigate the efficacy of an FDA approved selective agonist, MBG. MBG is able to mediate relaxation reversibly in contracting human myometrial tissue strips (Fig. 2). Although other β3AR agonists have been examined, (Arrowsmith et al 2010; Rouget et al. 2004), the effects of MBG shown here are unique. MBG is a Pregnancy Category C drug that could be employed during pregnancy. Since MBG is already FDA approved for the relaxation of the bladder smooth muscle, regulatory approval for its use in clinical trials for preterm labor in the future may be accelerated.

In order to incorporate MBG as a potential tocolytic we sought to confirm its selectivity and signaling in myometrium. The relaxation effects of MBG (Fig. 3)

were completely inhibited by the β3AR inhibitor, SR59230A, which confirms its selectivity for the receptor and partial inhibition of MBG induced relaxation was seen in the presence of either L-NNA or paxilline. Since the β3AR has been shown to be linked to the activation of eNOS (Dessy et al. 2004), partial inhibition from L-NNA showed that the receptor is linked to eNOS activity in the myometrium as well. Data supports the importance of eNOS in the myometrium during pregnancy as it decreases in concentration when at term or in labor (Fig. 1B). Paxilline inhibits the effects of the BK_{Ca}, confirming previous study findings on the coupling of this potassium channel with the β3AR in vascular tissue (Doheny et al. 2005). However, these data show that the inhibition of MBG-mediated relaxation is only partial and that relaxation is due to multiple downstream mechanisms. A combination of both paxilline and L-NNA completely inhibited the effects of MBG, similar to that of SR59230A confirming that MBG stimulates the β3AR in disparate cellular compartments in myometrium involving both eNOS and BK_{Ca} mediated pathways.

The potential of MBG targeting multiple compartments and the crucial role eNOS may play in managing quiescence lead us to investigate whether NO was being generated from β3AR stimulation in phMEC and phUSMC. Our data (Fig. 4A-C) confirm that microvascular endothelium and myocytes can be isolated and that the β3AR is present in both cell types. β3AR expression is greater in phUSMC versus phMEC (Fig. 4C). Using DAF-FM diacetate as a NO detector, we show that MBG increases NO production over a one-hour time period in phMEC but not in

phUSMC (Fig. 5). This data verifies that NO generation by the β3AR is specific to the microvascular endothelium.

As novel approaches to development of tocolytic strategies continue to be made, the β3AR becomes more of an appealing target (Fig. 7). The β3AR is ubiquitous in the myometrium throughout pregnancy. MBG relaxes the myometrium using multiple downstream mechanisms, involving both eNOS and BK_{Ca}. The β3AR was identified in both phUSMC and phMEC, while mediating NO generation only in phMEC.

Fig. 7

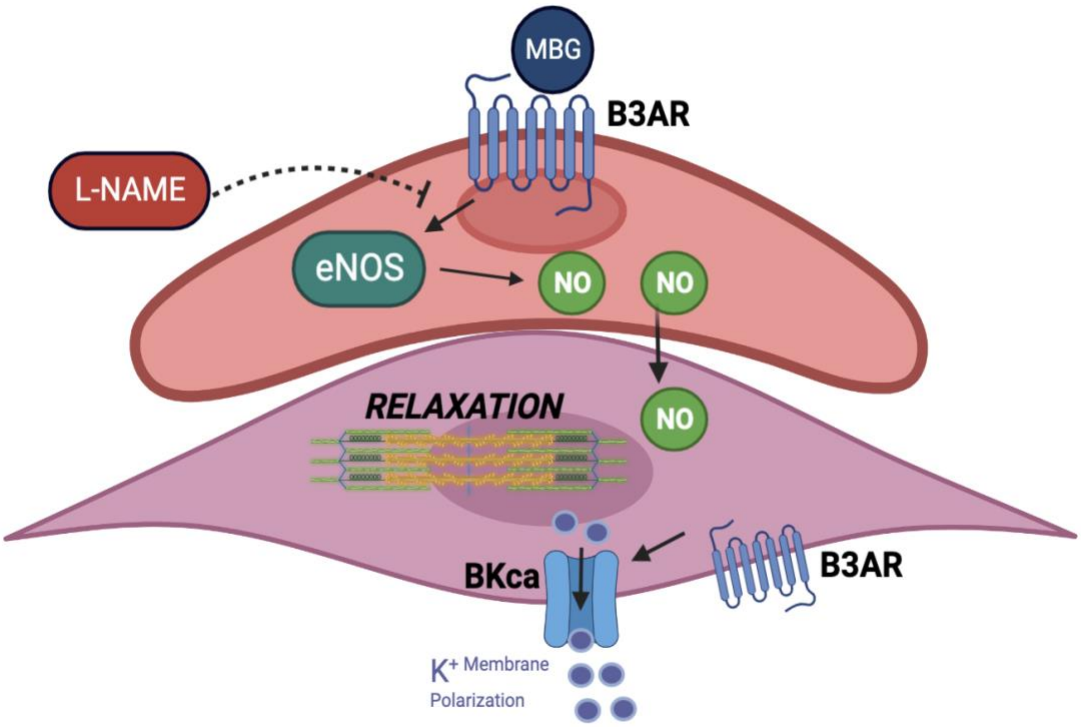

MBG
B3AR
L-NAME
eNOS
NO
NO
RELAXATION
NO
BKca
B3AR
K+ Membrane
Polarization

Figure 7: β3AR signaling in the human myometrium - β3AR stimulation from MBG activate eNOS to increase production of NO in phMEC to support relaxation in the myometrium. β3AR activates the BK_{Ca} to promote a polarized state in the myometrium.

Chapter 3

Modulating Connexin 43 activity by S-nitrosation and β3AR activation for relaxation of the human myometrium

Disclosure Statement

The authors declare they have no conflict of interest. Permission granted to use published work for dissertation (Scott D. Barnett, Asif, Anderson, et al. 2020). (Appendix A)

Abstract

Currently available tocolytics are ineffective at significantly delaying preterm birth. This is due in part to our failure to better understand the mechanisms that drive spontaneous preterm labor. Cyclic nucleotides are not the primary contributors to myometrial quiescence, but instead nitric oxide mediated protein S-nitrosation is integral to the relaxation of the tissue. Connexin-43, a myometrial 'contractile associated protein' that functions as either a gap junction channel or an hemichannel, was the focus of this study. Connexin 43 is S-nitrosated by nitric oxide, which correlates to an increase of connexin 43-pS368 (gap junction inhibition), as well as an increase in the hemichannel open-state probability (quiescence). Pharmacologic inhibition of Cx43 with 18β-Glycyrrhetinic acid exhibits a negative inotropic effect on the myometrium in a dose-dependent manner, as does administration of nebivolol, a nitric oxide synthase activator that increases total protein SNOs. β3 adrenergic receptor has been shown to activate tyrosine kinase, Src, which has been shown to down regulate connexin 43. Employing contractile studies, we have shown that inhibition of Src decreases the relaxation effects produced by mirabegron on contracting pregnant human myometrial tissue strips. Western blot analysis determined that Src expression was decreased in both preterm and term laboring myometrial tissue. Imaging revealed that the stimulation of β3 adrenergic receptor phosphorylates the Y265 site on connexin 43 (gap junction down regulation) in primary cells of pregnant human myometrium. Western blot analysis and immunofluorescent imaging indicate that

mirabegron decreases the expression of connexin 43. Here it is shown that modulation of connexin 43 through S-nitrosation and β3 adrenergic receptor show promise to developing tocolytic strategies.

Introduction

Seventy-five years of tocolytic development (Vanbésien and Eichner 1956) has produced little in terms of effective therapies to halt preterm labor (PTL) or delay preterm birth (PTB)(WHO 2015). This is concerning not only for the more than 15-million infants born prematurely each year, many of whom will experience lifelong health complications, such as chronic lung and cardiovascular disease, but also because PTL/PTB imparts in a substantial financial burden on the health care industry, costing greater than 40-billion annually (adjusted) in the United States alone(Behrman and Butler 2007a). It is clear that new strategies must be employed to mitigate this obstetric dilemma.

In contrast to most smooth muscle types, cyclic nucleotides (cGMP/cAMP) are not the dominant mediators of myometrial quiescence(K K Bradley et al. 1998; Lai, Tribe, and Johnson 2016; Scott D. Barnett et al. 2018). Interestingly, non-diseased myometrium will readily relax to nitric oxide (NO·), whose canonical action in most smooth muscle types lies in cGMP/PKG activation, but whose primary actions differ in the myometrium through protein S-nitrosation. It is well established that NO-mediated protein S-nitrosation (SNO) imparts a wide range of effects on the cell, and that the disparate regulation of SNOs correlates to dozens of diseases(Foster, McMahon, and Stamler 2003; S.D. Barnett and Buxton 2017). Protein S-nitrosations are not only dysregulated in the myometrium of sPTL patients(Craig Ulrich et al. 2012), but the functional effect of these SNOs on

contractile associated proteins (CAPs) underlies uterine quiescence(Scott D. Barnett et al. 2018).

Connexin-43 (Cx43) is a member of large family of connexins and pannexins which actively mediate cell-cell communication(Söhl and Willecke 2004), including the myometrium(Pierce et al. 2002). Cx43 functions as either a hemi-channel (HC), where it promotes quiescence through the permeation of prostaglandin-E_2(Burra and Jiang 2009) and other low molecular weight molecules(Hansen et al. 2014) from the cell, or as two such HCs in opposing membranes combining to form a gap junction channel (GJC), where it couples cells electrically to facilitate the propagation of contractile signals. The GJC/HC transition is largely driven by C-terminal phosphorylations(Leithe et al. 2018), and in some cell types HC and GJC permeation is altered by its S-nitrosation (SNO) state, particularly by nitrosation of cysteine 271 (C271)(Straub et al. 2011). Given that conditions promoting the Cx43 HC state and GJC inhibition may serve to facilitate myometrial quiescence, here we have elected to explore the functional consequences of S-nitroso regulation of Cx43 in human myometrium.

The β3 adrenergic receptor (β3AR) is a potential target for the development of novel tocolytic strategies due to its increased expression during gestation (Asif et al. 2022; Rouget et al. 2005). Initially, the β3AR was first discovered in 1989 and was found to be present in adipose tissue, vascular endothelium, and smooth muscle of the bladder and colon (Deeks 2018; Emorine et al. 1989). Then the β3AR was found in the myometrium, and identified to have a relaxative effect on contracting myometrium tissue (Rouget et al. 2005; Asif et al. 2022). Unlike its β2

adrenergic receptor counterpart, the β3AR lacks the sequence for recruitment of β-arresting, which prevents it from being downregulated from over activation (Schena and Caplan 2019).

The β3AR has multiple downstream signaling pathways to produce quiescent mediators. Some pathways that have been identified in mediating quiescence in the human myometrium include nitric oxide production in endothelial cells, BK_{CA} activation, and inhibition of oxidase activity in macrophages (Asif et al. 2022; Hadi et al. 2017). The β3AR can utilize either a G_s or G_i dependent pathway, to potentially recruit kinases, such as Src, to affect different contractile associated proteins (Hadi et al. 2013). The effect of Src on contracting myometrium has been unsure due to many Src inhibitors being nonselective and targeting many other kinases; however, the recent production of a highly selective Src inhibitor may help identify the downstream effect of Src in the β3AR pathway (Tatton et al. 2003; Brandvold et al. n.d.). The vast array of signaling pathways associated with β3AR questions whether contractile associated proteins, such as Cx43, can be affected to produce quiescent effects in the human myometrium.

The conspicuous dearth of tocolytic strategies is due in part to our collective failure to better understand the mechanisms that underlie the biochemical regulation of uterine smooth muscle relaxation. Both the cGMP-independence of myometrial relaxation to NO(K K Bradley et al. 1998), and the dysfunction of preterm laboring myometrium to NO-mediated relaxation(Barnett et al. 2018), are known. What drives spontaneous PTL is not, but Cx43 is known to propagate contractions in the induced laboring state. The importance of NO-mediated protein

S-nitrosation in the myometrium during pregnancy, coupled with the multiple modulation sites on Cx43 as a myometrial contraction-associated protein (CAP), makes Cx43 an attractive target of study. Here we investigate the regulation of Cx43 during pregnancy/parturition, the effect of NO and β3AR stimulation on Cx43 function, as well as the tocolytic potential for therapeutics that mediate Cx43 activity.

Materials and Methods:

Tissue collection: Human tissue collection has been carried out in accordance with the Declaration of Helsinki and approved by the Institutional Review Board at the University of Nevada Biomedical Review Committee for the protection of human subjects. Human uterine biopsies were obtained with written informed-consent from mothers with singleton pregnancies undergoing Cesarean section without known infection or rupture of membranes (PROM/PPROM), as previously described(Barnett et al. 2018). All experiments were performed in accordance with the NIH guidance on the use of human tissues in research. Exclusion criteria include age < 18 years, any history of drug abuse, co-morbid diagnoses such as HIV infection or AIDS, hepatitis C infection, uncontrolled diabetes, renal disease, preeclampsia, IUGR, placenta previa and any use of steroids other than betamethasone including topical use during pregnancy. SARS-CoV-2 positive patients were also excluded from this study. Tissues were transported to the laboratory immediately in cold Krebs buffer containing, in mM: NaCl (118), KCl (4.75), CaCl2 (2.5), KH2PO4 (1.2), NaHCO3 (25), MgCl2 (1.2), dextrose (20), and adjusted to pH 7.4. Tissues were dissected under magnification to isolate smooth muscle, employed in contractile experiments or snap frozen in liquid nitrogen (LN_2), and stored at -150 °C. Pregnant laboring patients ranged from 39 to 41 wk. gestation, with the mean at 39 wk. Preterm laboring patients without

evidence of infection, PROM or preeclampsia ranged from 29.2 to 36 wk. of gestation, with the mean being 33.6 wk.

Contractile Studies: The dissected myometrium strips are placed in 4-channel tissue bath systems (Danish Myo Technology 820MS, US), while submerged in oxygenated (95% O_2 and 5% CO_2) Krebs buffer at 37^{o}C. Strips are attached to transducers that were pulled at 1 gram of tension for β3AR related experiments and 2 grams of tension for Cx43 related experiments. The strips are then submerged in 60mM KCl for 3 minutes, and then followed by a washout to induce spontaneous contractions. After a one-hour equilibration period and the induction of spontaneous contractions, tissues were dosed with 8nM oxytocin to stimulate maximum contractile effects. TAT-Gap19 was tested as a single dose at 100 µM (or buffer vol. equivalent) for 60 minutes in KCL-stimulated (60 mM, 3 minutes) tissue without the addition OT (n=3, 2 strips per patient). After maximum contractile effects have equilibrated, tissues were treated with a combination of selective Src inhibitor, KB Src4 (15 minutes, 4660, Tocris, US), ± β3AR agonist, mirabegron (MBG, 30 minutes, 7070, Tocris, US), or in combination with nebivolol and 18β-glycyrrhetinic (18β-GA) acid. Control tissues were dosed with volume equivalent DMSO. Washouts were performed after dosing periods to confirm a return in contractions. In overnight contractile studies, after oxytocin tissues were treated with mirabegron in Gibco Dulbecco's Modified Eagle Medium, while maintaining tension for 24 hours in a 37^{o}C incubator. Tissue strips were then submerged back in Krebs buffer and retreated with KCl and oxytocin. Voltage changes in contractions are recorded to digital signals by the transducers and

transferred to the LabChart software for analysis (version 8.1.12, ADInstruments, Australia) (Asif et al. 2022; Barnett et al. 2022). Tissues that did not respond to KCl challenge or failed to produce post washout contractions at the end of the treatment period were not included into the analysis.

Western Blot: Protein was isolated in MAPK buffer containing, in mM: Tris-HCL pH 6.8 (60), glycerol (1%), SDS (2%), leupeptin (0.001), EGTA (1) EDTA (1), AEBSF (1), Na_3VO_4 (1), and protease/phosphatase inhibitors (PPC110: Sigma Aldrich, St. Louis, MO). Samples were crushed using LN_2 w/ mortar and pestle, followed by 5 minutes in a tissue homogenizer. Sample concentrations were determined using EZQ (R33200: Thermo Fischer, Waltham, MA). Note - 'n's were not equal for all conditions due to an scarcity of samples, particularly PTL.

For Cx43 in disparate states of pregnancy, 40 µg of protein lysate was run at 200 V for 45 min on a 4-20% PAGE gel and transferred to nitrocellulose, blocked in Licor® blocking buffer. The Western blot was labeled with mouse anti-Cx43 polyclonal 1° (1:1000, CX-1B1: Thermo Fischer, Waltham, MA), followed by either Alexa Fluor 680-donkey anti-mouse 2° (1:25k, ab175774: Abcam, Cambridge, MA) or IRDye 800-donkey anti-mouse 2° (1:25k, ab216774: Abcam, Cambridge, MA). Cx43 expression was normalized to rabbit anti-GAPDH (1:1000, 2118S: Cell Signaling Technology, Danvers, MA) followed by either Alexa Fluor 680-goat anti-rabbit 2° (1:25k, ab216773: Abcam, Cambridge, MA) or IRDye 800-donkey anti-Rabbit (1:25k, 926-32213: Licor Biotechnology, Lincoln, NE). For pS368:S368 blots we used rabbit anti-pS368-Cx43 1° (1:1000, ab30559: Abcam, Cambridge, MA) and IRDye 800-goat anti-rabbit 2° (1:25k, ab216773: Cambridge, MA),

followed by mouse anti-Cx43 polyclonal 1° (1:1000, CX-1B1: Thermo Fischer, Waltham, MA) and Alexa Fluor 680-donkey anti-mouse 2° (1:25k, ab175774: Cambridge, MA). For S-nitrosated proteins gels were run as described above with 30 µl of eluted total-SNO proteins (see biotin switch method) with either a reversible 680 nm total proteins stain, Licor Revert™ (p/n 926-11010: Licor, Lincoln, NE), or with the Cx43 antibody described above.

For Src expression and MBG treated cells, protein lysates were derived from both tissue pieces and primary cell culture. Tissue lysates were made similarly as listed above. Cells were collected via cell scraper after the addition of MAPK buffer and then sonicated. For Src and Cx43 expression in tissue lysates and cell lysates, 40µg and 25µg of protein respectively was loaded into a 4-15% Mini-Protean TGX precast gel in standard tris/glycine PAGE buffers (4561081, BIO-RAD, US), ran at 200V, and then transferred to nitrocellulose (1704158, BIO-RAD, US) followed by being blocked in Licor blocking buffer for at least one hour. Western blot was labeled with either primary mouse anti-src (1:1000, sc-5266, Santa Cruz, US) or primary mouse anti-connexin 43 (1:1000, CX-1B1, Thermo Fisher Scientific, US) for overnight incubation, followed by a one-hour incubation of secondary antibody Alexa-Fluor 680 donkey anti-mouse (1:15000, ab175774, Abcam, US). Both proteins of interest were normalized to their respective GAPDH concentrations using the primary rabbit anti-GAPDH (1:1000, 2118S, Cell Signaling Technology, US) and the secondary IRDye 800 donkey anti-rabbit (1:15000, 926-32213, Licor Biotechnology, US).

Biotin Switch and Streptavidin Pulldown: Myometrial tissue samples (n=4 patients) were hung in a tissue bath (see *contractile studies*) and exposed to either oxytocin (8 nM, 3 strips per patient) or oxytocin followed ~10 minutes later by GSNO (300 µM, 3 strips per patient) at the start of a contraction, then snap frozen with LN_2 at either peak contraction or maximal relaxation and stored in amber tubes (to protect S-nitrosation) at -150°C. Proteins were initially isolated in MAPK buffer with 2% SDS (see *western blot*). For the biotin switch(Jaffrey and Snyder 2001), 1397 µg of total protein per sample was then suspended in HEN buffer containing, in mM: HEPES (25), EDTA (1), neocuproine (0.1), pH 7.7. An additional 2.5% SDS (final) and 30 mM N-ethylmaleimide (NEM) were added to block free thiols. Samples were incubated at 50°C in the dark for 20 minutes with frequent vortexing. (3) volumes of -30°C 100% acetone were added to each sample, and proteins were precipitated at -30°C overnight and collected by centrifugation at 3,000xg for ten minutes. After rinsing with 70% acetone protein pellets were dried using dry Nitrogen, then re-suspended in HENS (HEN + 1% SDS) with 10 mM ascorbate (to reduced SNO proteins) and 0.25 mg/ml biotin-HPDP (No. 16459: Cayman Chemical, Ann Arbor, MI) and rotated at r.t. for 1 hour. Proteins were precipitated and rinsed as before, and resuspended in a mixture of HENS (25%, 87 µl), neutralization buffer (50%, 174 µl) containing, in mM: HEPES (25), NaCl (100), EDTA (1), 0.5% Triton X-100, pH 7.5, and 75 µl of magnetic streptavidin beads (spherotech svmx-10-10, Lake Forest, IL). Samples were washed 5 times with (10) volumes of neutralization buffer in a magnetic stand, then recovered in 35 µl elution buffer containing a 1:10 dilution of HENS/H_2O with 1% v/v β-mercaptoethanol.

Note - two proteins pellets from GSNO treated samples were lost during a rinse phase and excluded from the data set.

Cell Culture: Primary cells from term non-laboring (TNL) tissue were cultured as previously discussed (Barnett et al. 2022; Asif et al. 2022). 20mg collagenase (CLS2. Worthington, US), 10mg Trypsin (27250-018, Gibco, US), and 10 ml of solution containing 5 ml of MACS buffer and 5 ml of Gibco Dulbecco's Modified Eagle Medium (DMEM, 11995-065, Gibco, US) are mixed and incubated at 37^{o}C with small pieces of dissected myometrium in three, 90 second agitation steps. This is followed by two 45-minute incubation periods at 37^{o}C with rotation. The resulting digestion is triturated three times and filtered through a 100-micron sterile mesh. The cells from this are grown to 80% confluency and preincubated with FcR blocking reagent, then separated over CD31+ bead LS columns over a MidiMACS separator (130-042-302, Miltenyi BioTec, US) into pregnant human uterine smooth muscle cells (phUSMC) and pregnant human myometrial endothelial cells. The phUSMC were cultured in DMEM combined with 50 U/ml streptomycin, 50 µg/ml penicillin, and 10% FBS and supplemented with estrogen (15 ng/ml) and progesterone (200 ng/ml). At 80% confluency in P0 culture, phUSMC are grown to P3 and either used in experiments or frozen at 1x10^6 cell/ml.

Human Embryonic Kidney (HEK293, p10-p25 - ATCC, Manassas, VA) and telemorized pregnant human uterine smooth muscle cells (hTRT-phUSMC), p20-p30 - created in-house(Heyman et al. 2013)) were grown in Dulbecco's modified Eagle's medium (DMEM) with 50 U/ml streptomycin, 50 µg/ml penicillin, and 10%

FBS. phUSMC cells were further supplemented with estrogen (15 ng/ml) and progesterone (200 ng/ml). All cells were cultured in a balanced oxygen (95% O_2, 5% CO_2) 37°C incubator.

Ethidium Bromide Assay: HEK293 and hTRT-phUSMC cells were seeded (~2500 cells/well) in 96-well plates (94.6000.024, Lumox Multiwell: Sarstedt, Newtown, NC) and grown to ~50-80% confluency. Experimental protocol was modified from(Contreras et al. 2003). 24-48 hours after seeding the medium was exchanged for HEPES-buffered Krebs-Ringer (-) Ca^{2+} solution containing, in mM: NaCl (120), KCl (5), $MgCl_2$ (1), $NaHCO_3$ (25), HEPES (5.5), and D-glucose (1.1), pH 7.4, ± $CaCl_2$ (2.5, as dictated by experiment). 10 µM ethidium bromide (EtBr) was added to each well ± GSNO (300 µM) or TAT-Gap19 (100 µM, a membrane permeable Cx43 HC inhibitor) then returned to the cell culture incubator for 5 minutes. Following this, all wells were rinsed 3x with 37°C HEPES-buffered Krebs-Ringer (-) Ca^{2+}. This was denoted at t=0, and the plates were returned to the incubator. At each experimental time point (0,30,60, or 90 minutes), treated cells were exposed to paraformaldehyde to halt progression of EtBr intercalation, then rinsed 3x with buffer. Each well was then treated with DAPI (for cell counts), followed by 3x rinses. Plates were imaged on an ImageExpress® Nano (Molecular Devices, San Jose, Ca) at 4x using MetaExpress® software (v.6.5.4.532) at a fixed exposure time of 1500 ms. Data collection and analysis was limited to a square in the middle of each well covering 32.63% with an average of 1024 cells to avoid incorporation of anomalous growth patterns common near the periphery of plates. Plate images in Figure 3 are magnified 6.8x (300x300px) and false colored (blue,

DAPI: green, EtBr) to better visualize EtBr uptake, but were analyzed using the full scan with ImageJ (v. 2.0.0-rc-69/1.52p). Cell counts were determined by setting an appropriate threshold, followed by “Analyze Particles” (size=100-Infinity) on the DAPI channel. EtBr uptake was determined by “area fraction” of the EtBr channel using fixed threshold to avoid altering the analyzed signal from well to well. Area fractions were normalized to cell count, then each normalized area fraction was further normalized to the area fraction of the negative control [(-) EtBr] at each time point to account for noise in the background signal. t=0 was set to ‘zero’ and data is displayed as a fold-increase in fluorescent signal from t=0.

Immunofluorescence: The phUSMC from the CD31+ bead separation were plated on 35mm glass bottom dishes (P35G-0.170-14-C, MatTek Corporation) and grown to 70-90% confluency. Cells were treated with mirabegron (30μM) ± 15-minute preincubation of KB Src 4 (30μM) for one hour. Cells are then washed three times with 1x PBS and incubated with 4% paraformaldehyde for 15 minutes, followed by 0.5% triton-x for 5 minutes, and 5% BSA blocking buffer for 1 hour with three PBS washes between each step. Cells were then stained with primary rabbit anti-phospho Y265 connexin 43 in 1% BSA (1:100, PA5-104561, Thermo Fisher Scientific, US), followed by secondary Alexa Fluor 594 (ab150080, Abcam, US). Wheat germ agglutinin (WGA) conjugated to Alexa Fluor 488 (W11261, Thermo Fisher Scientific, US) was used to define cell membrane. DAPI mounting medium (H-1500, Vector Laboratories, US) was used to stain the nucleus of the cells. All images were taken with negative controls (lack of primary antibody) to confirm there was no nonselective binding (data not shown).

Transfection: First, 20ng of GJA1 - HaloTag human ORF in pFN21A (FHC03362, Promega, US) added to chilled E. coli Top 10 chemically competent cells (C404010, Thermo Fisher Scientific, US) are placed in 42^{o}C water bath for exactly one minute, and then placed back on ice with Luria-Bertani (LB) medium, followed by a one-hour incubation in 37^{o}C water bath. The resulting cell mixture was applied onto a room temperature agar plate [1.5% agar, LB (20g/L), and ampicillin (100μg/ml, 69-52-3, Sigma-Aldrich, US)], and placed upside down into a 37^{o}C incubator overnight. One colony from the agar plate was placed into LB medium with ampicillin (100μg/ml), and incubating overnight at 37^{o}C while shaking. Isolated the plasmid after cloning using a Zyppy plasmid miniprep kit (D4036, Zymo Research, US), followed by quantification using a nanodrop ND-1000 (Thermo Fisher Scientific, US). phUSMC were cultured and grown to 70-90% confluency. A 1:1 ratio of DNA master mix [Opti-MEM (31985062, Thermo Fisher Scientific, US), 5μg of DNA plasmid, P3000 reagent (2μl/μg of plasmid, 2471954, Invitrogen, US)] and solution containing Opti-MEM with 5% lipofectamine 3000 (2471954, Invitrogen, US) were added to phUSMC and incubated for 72 hours.

Pulse-Chase: Pulse-chase experiment using HaloTag ligands were performed using protocols established by Promega. The phUSMC transfected with GJA1 - HaloTag human ORF in pFN21A (FHC03362, Promega, US) were washed with 1x PBS and replaced with phenol red free DMEM (21,063,029, Thermo Fisher, Waltham, USA) containing 50 U/ml streptomycin, 50 μg/ml penicillin, and 10% FBS and supplemented with estrogen (15 ng/ml) and progesterone (200 ng/ml). Cells were then dosed with TMR HaloTag Ligand (5μM, G8251, Promega, US) for 15

minutes, followed by three washouts with PBS and media replacement. Cells incubated in media for one hour to allow remaining ligand to efflux out of the cell, followed by another washout and media replacement. Images were taken at 10x (ECHO, US) to confirm the presence of ligand in cells transfected with GJA1 – HaloTag, and compared to a negative control of phUSMC that were given ligand without transfection. Once transfection is confirmed, cells are then incubated with either 30μM mirabegron or volume equivalent of drug solvent for one hour. Cells are then washed in 1x PBS and fixed with 4% paraformaldehyde (15 minutes), followed by the addition of DAPI mounting medium (H-1500, Vector Laboratories, US). Images were taken at 10x (ECHO, US).

Statistical Analysis: For contractile studies, each 'n' is a different patient from a TNL sample obtained from tissue collection. Each patient had multiple tissue strips tested, but they still accumulated as a single sample. Contractions were analyzed by calculating the integral relative to the minimum baseline for each respective contractions to measure the area under the curve (AUC), and by calculating the difference between the highest and lowest peaks of each respective contraction to measure the peak force. The last three contractions in the last 15 minutes of each dosing period were analyzed. Each measurement for tissue strips were double normalized to their respective oxytocin baseline and then to a control tissue strip. Traces were made by normalizing the maximum and minimum values of the baseline for each condition to 1 and 0, respectively, and then normalizing the dosing period data points based off those values. For the 24-hour contractile experiments, control and experimental data was shown as a percentage compared

to their baselines. For western blot experiments, each sample represented a different patient. Statistical analysis for contractile studies and western blots were done using two-tailed, unpaired t-tests through Prism (v. 8.4.3, Graphpad Software, US) with significance showing at $p<0.05$.

Results

Cx43 Protein Expression in sPTL

A multitude of genes and proteins are differentially regulated in women who experience preterm labor(Knijnenburg et al. 2019; Paquette et al. 2018a). We previously determined that s-nitrosoglutathione reductase (GSNOR), a negative modulator of NO availability, is upregulated in sPTL myometrium, which decreases the availability of NO and reduces total protein S-nitrosations(Scott D. Barnett et al. 2018). Here we sought to determine if Cx43 is also dysregulated in sPTL myometrium.

In human tissue we found, relative to NP myometrium (n=14), Cx43 expression was significantly higher in all pregnancy groups (PTNL P=0.0033, n=6, 32.0wk ± 2.32; TL P<0.0001, n=14, 39.0wk ± 0.44; TNL P<0.0001, n=14, 38.8wk ± 0.67), with the notable exception of sPTL (sPTL P=0.8411, n=6, 33.64wk ± 3.25) (Fig.1).

Effect of NO on Cx43 Phosphorylation & S-nitrosation

The expression of Cx43 in the myometrium is only one of several important metrics when establishing its role in contractile dynamics. It is well-known that Cx43's phosphorylation state, particularly at serine-368 (S368) on the c-terminus, is critical to GJC (contraction) function(Lampe et al. 2000; Solan and Lampe 2014). Because myometrium is known to relax to NO independent of the canonical cGMP-PKG pathway(I. L. Buxton 2004), we sought to determine whether NO would facilitate an increase in Cx43 O-phosphorylation at S368 (pS368). Using an *ex vivo* organ bath, we attached human myometrial tissue strips to a force transducer in a

Fig. 1

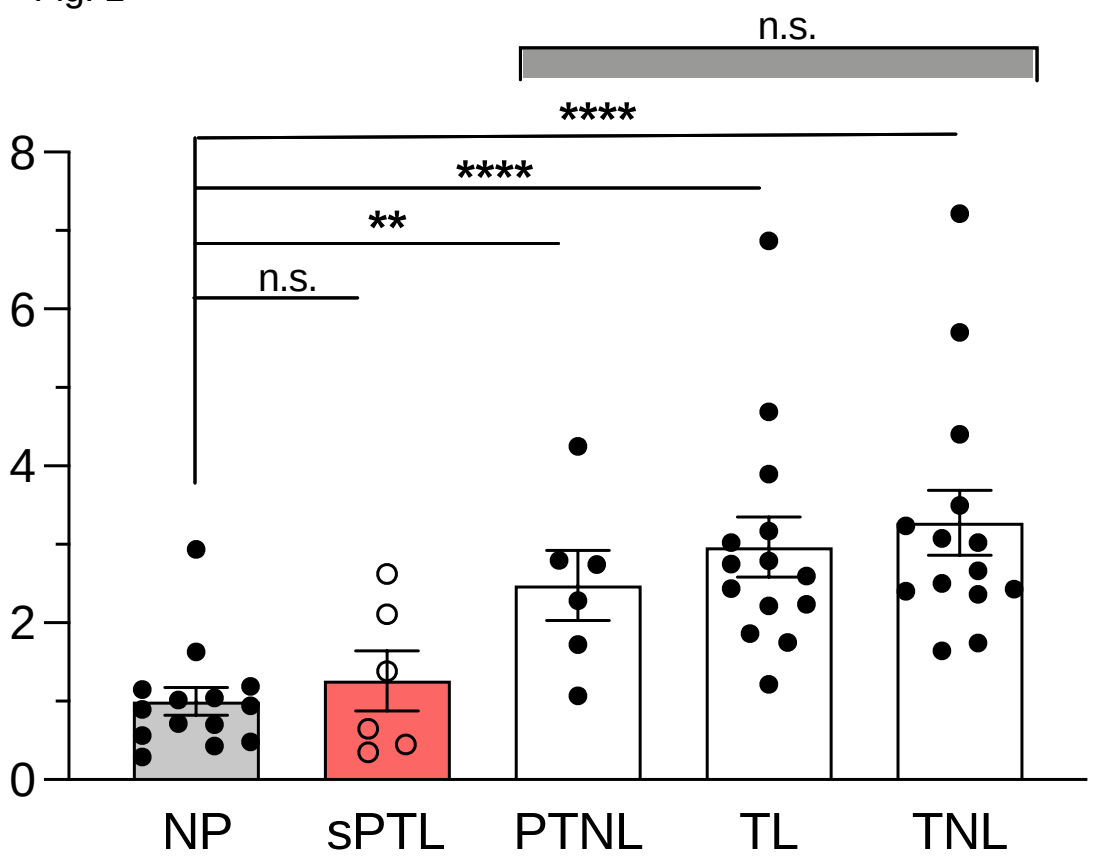


Figure 1: Cx43 is dysregulated in sPTL myometrium - Human myometrial expression of Cx43 increases by approximately 2.5-fold in PTNL tissue (P = 0.0033) and holds steady to term (TL: P<, 0.0001, TNL: P<, 0.0001); however, sPTL myometrial expression of Cx43 is not significantly different than NP tissue (P = 0.8411), implying a dysregulation of its expression. n.s., not significant.

physiological buffer, then exposed the tissue to either oxytocin (8 nM), after which it was snap-frozen with LN_2 (spray gun) at peak contraction, or to oxytocin, followed NO (GSNO, 300 µM), then snap-froze at maximal relaxation (TNL n=4, 3 samples per 'n'). Total protein lysate was extracted from the samples and the ratio of pS368:S368 was analyzed *via* western blot (Fig 2A). We found that the ratio of pS368:S368 was ~2.4-fold higher (P=0.0281) in myometrium treated with NO as compared to tissue treated with OT, intimating a role of NO in Cx43-mediated quiescence.

In addition to phosphorylation, we examined Cx43 S-nitrosation, a posttranslational modification that acts as a critical mediator of protein function in the myometrium. Total protein S-nitrosation in the myometrium can vary wildly based on the pregnancy state, in particular in women who experience sPTL(Craig Ulrich et al. 2012). Moreover, this posttranslational modification is known to affect contractile dynamics(Barnett et al. 2018). S-nitrosation of Cx43 has been shown in other tissue types to alter the protein's function(Lillo et al. 2019; Retamal et al. 2006); therefore, we sought to determine whether NO-mediated phosphorylation of Cx43 at S368 correlates with Cx-43 S-nitrosation. Using the same protein lysates as above, we performed a biotin switch assay to identify whether Cx43 was S-nitrosated when exposed to NO (300 µM). We found that SNO-Cx43 levels increased 2.08-fold in NO-treated samples (P=0.0046) over OT treated samples (Figure 2B), while total protein SNOs increased 1.37-fold (P=0.0274, Figure 2C).

Effect of NO on Cx43 HC Function

Fig. 2

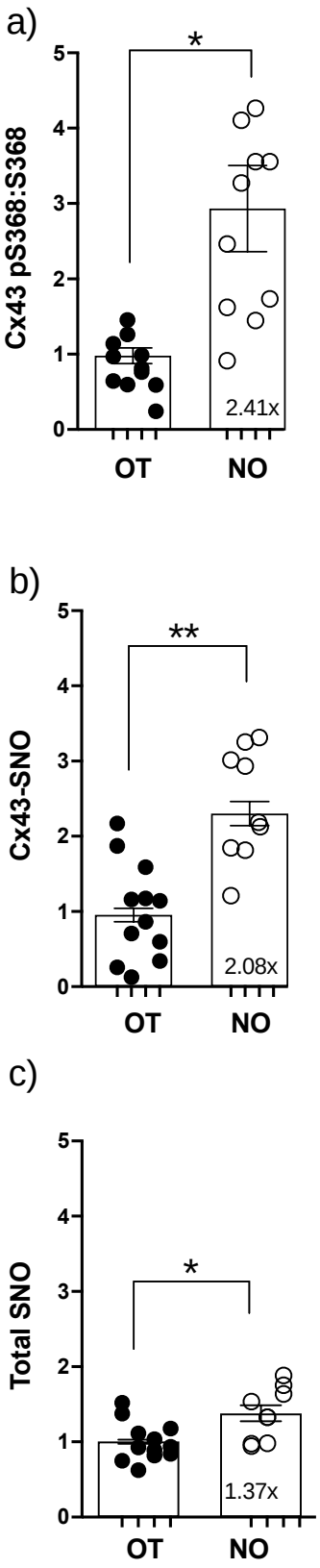

a)
Cx43 pS368:S368
*
2.41x
OT
NO
b)
Cx43-SNO
**
2.08x
OT
NO
c)
Total SNO
*
1.37x
OT
NO

Figure 2: Cx43 S-nitrosation promotes GJC inhibition (Cx43-pS368) in human myometrium - Human myometrial tissue (TNL) was hung in an organ bath and exposed to either OT or NO. (A) The ratio of pS368, a posttranslational modification that promotes GJC inhibition (quiescence), was approximately 2.4-fold higher in NO-treated tissue (P = 0.0281), (B) which also correlated to approximately2-fold increase in Cx43 S-nitrosation (P = 0.0046). (C) Total protein S-nitrosation in the tissue lysate also increased by approximately1.4-fold over OT-treated tissue (P = 0.0274).

After determining that NO correlates to both and increase in the pS368:S368 ratio and Cx43 S-nitrosation, we next sought to determine whether NO would directly affect its function. This was accomplished through an ethidium bromide (EtBr) assay(Contreras et al. 2003), which is measured as an increase in fluorescent signal as EtBr intercalates with nucleotides after passing though Cx43 HCs in the membrane. Telemorized pregnant human uterine smooth muscle cells (hTRT-phUSMC), and HEK293 cells, which show no appreciable Cx43 expression (Fig. 3C), were grown to ~50-80% confluence (n=3, ~3500 cells per well) and exposed to 10 µM EtBr for 5 minutes in Krebs-ringer buffer (-) Ca^{2+}. Following exposure, the experimental buffer was exchanged for fresh Krebs-ringer buffer (-) Ca^{2+} (t=0) and placed in a 37°C incubator (95%:5%, O_2:CO_2). At each time point (t=0,30,60,90) treated cells were washed and fixed with paraformaldehyde to halt the reaction. Cells were then imaged at 4x and the relative fluorescent increase (normalized to total cell count by DAPI labeling) over t=0 was observed. A higher fluorescent signal infers a prolonged open-probability of the channel during the EtBr loading phase. Under baseline conditions relative fluorescent signal increased by 4.58-fold (± 0.734) at t=90 (P=0.0089, Fig. 3A), which was completely eliminated by the use of 100 µM TAT-Gap19 (P=0.700), a membrane permeable inhibitor selective to the HC(Abudara et al. 2014). The addition of NO (GSNO, 300 µM) increased the fluorescent signal by 3.36-fold (± 0.698) over t=0 (P=0.0365); however, at 90 min GSNO treatment did not increase the fluorescent signal above baseline conditions at 90 min (P=0.2963). To determine if this occurred because the assay was run in Ca^{2+}-free buffer, which facilitates an HC

Fig. 3

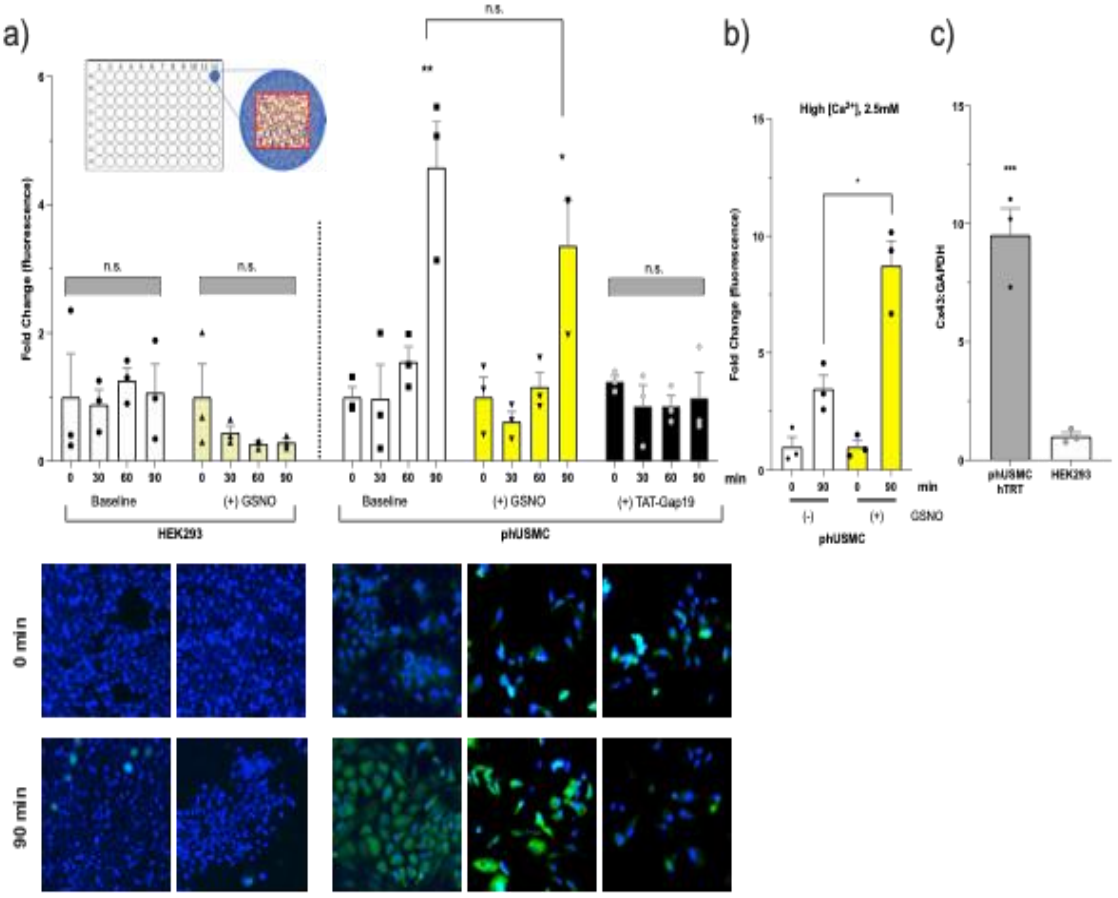

a)
b)
c)
Fold Change (fluorescence)
n.s.
**
Baseline
(+) GSNO
(+) TAT-Gap19
HEK293
pHUSMC
min
High [Ca2+], 2.5mM
(-)
(+)
GSNO
Cx43:GAPDH
pHUSMC hTRT
HEK293
0 min
90 min

Figure 3: Nitric oxide promotes Cx43 HC open state: EtBr selectively permeates Cx43 channels - (A and B) Human myometrial or HEK293 cells were exposed to EtBr 6 GSNO or TAT-Gap19 (HC blocker) in a Ca2+-free buffer for 5 minutes and then washed out. (A) Under baseline conditions (P = 0.0089) and with GSNO (P = 0.0365), EtBr uptake through HCs was significantly higher by 90 minutes in myometrial cells but not in those exposed to TATGap19 (P = 0.700), whereas no appreciable uptake of EtBr was observed in HEK293 cells (+) GSNO (P = 0.2533) or (2) GSNO (P = 0.8621). (B) Under 2.5 mM Ca2+, which increases the closed-state probability of Cx43, fluorescent signal in myometrial cells exposed to GSNO increased significantly over baseline treatment at 90 minutes (P = 0.0119). (C) 300 x 300–pixel magnification of 4x acquired images represented in (A) at t = 0 and t = 90, wherein cellular nuclei are false-colored blue (DAPI) and EtBr is green (Cx43). (D) Western blot data depicting the absence of Cx43 in HEK293 cells relative to telomorized (human telomerase reverse transcriptase - hTRT) uterine smooth muscle cells. n.s., not significant.

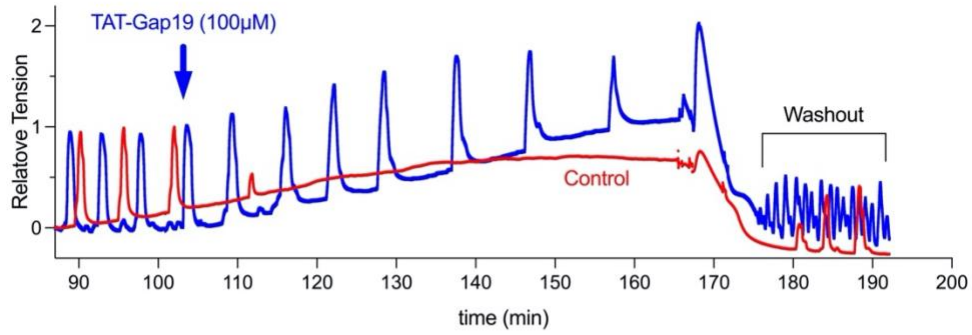
open-channel state(H. Li et al. 1996), we evaluated the effect of physiological extracellular Ca^{2+} (2.5 mM), which closes the HC(Ek-Vitorín, Pontifex, and Burt 2018). We found that the addition of NO (GSNO, 300 μM) significantly increased EtBr uptake over untreated baseline at 90 min (P=0.0119, Fig. 3B). The fluorescent signal in HEK293 cells, which do not appreciably express Cx43 protein (Figure 3C), was unchanged throughout the 90-minute observation period in both the untreated (ANOVA, P=0.8621) and NO-treated (ANOVA, P=0.2533) HEK293 cells (Figure 3A).

Cx43 HC inhibition and Tocolysis

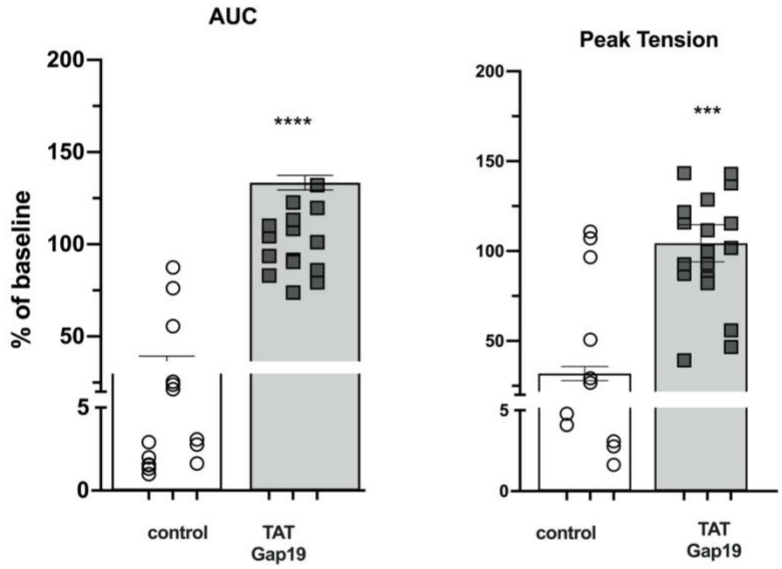
It is well established that Cx43 is a critical facilitator of coordinated muscle contraction, so here we consider whether or not Cx43 inhibition exhibits useful tocolytic properties. Two Cx43 inhibitors were selected for their actions on the GJC and HC: (1) 18β-Glycyrrhetinic acid (18β-GA), a compound found in the roots of *Glycyrrhiza glabra L.* (black liquorice) that inhibits GJC and HC; and (2) TAT-Gap19, a HC-selective inhibitor consisting of a nine-amino acid peptide fragment from the Cx43 cytosolic loop which contains a TAT-modification (transactivating transcriptional activator from human immunodeficiency virus 1; Figure 4) that greatly increases cell permeability and action on the channel(Abudara et al. 2014). Based on our previous data we posited that 18β-GA would blunt contractions by preventing the propagation of the contractile signal between uterine smooth muscle cells, while TAT-Gap19 would most likely confer positive inotropic effects by inhibiting normal HC function.

Fig. 4

a)


TAT-Gap19 (100μM)
Relatove Tension
2
1
0
Washout
Control
90
100
110
120
130
140
150
160
170
180
190
200
time (min)

b)


AUC
% of baseline
200
150
100
50
5
0
****
control
TAT
Gap19
Peak Tension
200
150
100
50
5
0
***
control
TAT
Gap19

c)

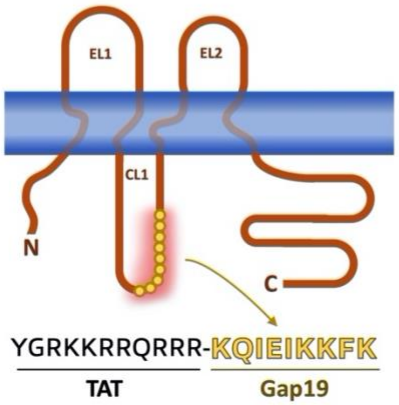

EL1
EL2
CL1
N
C
YGRKKRRQRRR-KQIEIKKFK
TAT
Gap19

Figure 4: Cx43 HC inhibition promotes myometrial contractions - Human myometrial tissue (TNL, n = 4) was KCl-challenged (60 mM, 3 minutes) in an organ bath to initiate contractions but was not exposed to oxytocin. (A) Tissue treated with TAT-Gap19 (100 mM), a Cx43 HC-selective inhibitor, maintained contractions throughout the experimental period, whereas control tissue lost all contraction by approximately 100 minutes (contraction returned upon second KCl challenge after washout). (B) In the control tissue AUC decreased by approximately 80% as compared with TATGap19–treated tissue (P<, 0.0001) and peak tension falling approximately 69% (P = 0.005). (C) TAT-Gap19 consists of a nine-amino-acid cytosolic loop fragment of Cx43 and a linked N-terminal TAT modifier, which increases membrane permeability and enhances its inhibitory effect.

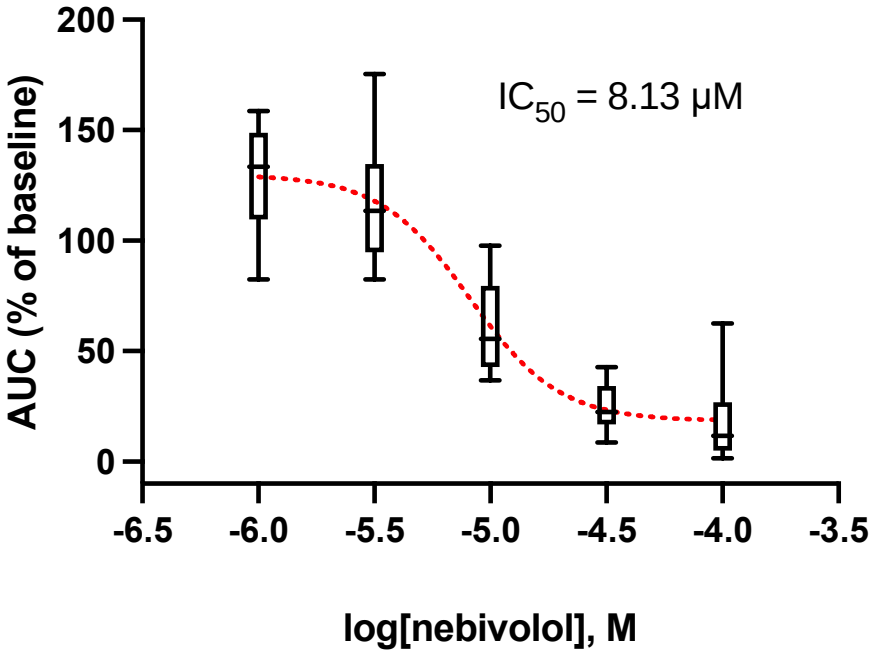
TAT-Gap19 was tested as a single dose at 100 μM (or buffer vol. equivalent) for 60 minutes in KCL-stimulated (60 mM, 3 minutes) tissue without the addition OT (n=3, 2 strips per patient). We hypothesized that TAT-Gap19 would promote a contractile state through HC-specific inhibition. Contractions from TNL myometrium that is not treated with OT will run down (data not shown); therefore, we were interested in determining if HC inhibition would promote contractions in tissue that would otherwise become quiescent. All TAT-Gap19 treated tissue maintained regular contractions throughout the dosing period (Figure 4A), while all but one control tissue ran down completely (contractions returned in all control samples following KCL-stimulation after 60-minute dosing period). Both AUC ($P<0.0001$) and peak tension ($P=0.005$) were significantly higher in TAT-Gap19 treated tissue (Figure 4B).

Co-administration of a Cx43 GJC Inhibitor and eNOS activator

A primary concern when administering any tocolytic are the potential off-target effects on the fetus and mother. Fetal toxicity not only limits which drugs may be used, but also at which concentrations. Because of this we propose that the co-administration of two or more drugs at modest doses may provide a complementary effect, particularly when each drug targets a known pathway associated with sPTL. We previously generated a limited data set in which nebivolol, an eNOS activator, displayed tocolytic properties(Barnett and Buxton 2018). Here we expanded upon that investigation and sought to establish whether or not the co-administration of nebivolol and 18β-GA, an Cx43 inhibitor, exerts enhanced negative inotropic effects.

Fig. 5

a)


200
150
100
50
0
AUC (% of baseline)
IC50 = 8.13 µM
-6.5
-6.0
-5.5
-5.0
-4.5
-4.0
-3.5
log[nebivolol], M

b)

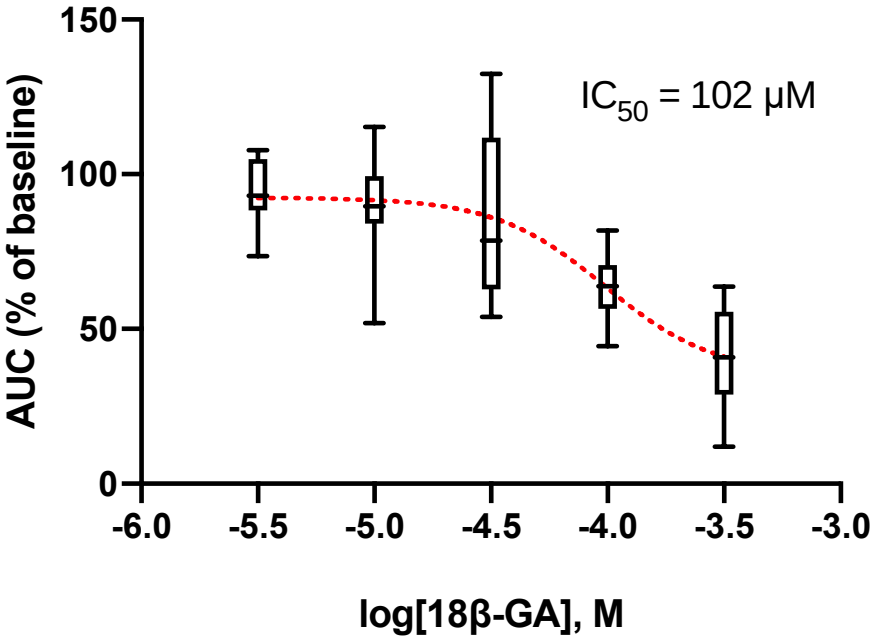

150
100
50
0
AUC (% of baseline)
IC50 = 102 µM
-6.0
-5.5
-5.0
-4.5
-4.0
-3.5
-3.0
log[18β-GA], M

Figure 5: Myometrial IC50 values of nebivolol and 18b-glycyrrhetinic acid - Nebivolol is known to increase the generation of nitric oxide through eNOS activation, whereas 18b-GA blocks Cx43 GJCs. TNL myometrium was hung in an organ bath and exposed to either (A) 1, 3, 10, 30, or 100 mM nebivolol (n = 3) or (B) 3, 10, 30, 100, or 300 mM 18b-GA (n = 5) for 1 hour. Calculated IC50 values are based on the AUC of the last three contractions of the dosing period prior to washout and were 8.13 and 102 mM for nebivolol and 18b-GA, respectively.

To begin, we experimentally derived IC_{50} values for nebivolol (Fig. 5A) and 18β-GA (Figure 5B) using TNL myometrium (log_{drug} vs. response) with the organ bath technique described above. We determined that the IC_{50} for nebivolol, based on AUC, was 8.26 μM (n=3 patients), while 18β-GA 102 μM (n=5 patients).

Because nebivolol acts on endothelial beta-3 adrenergic receptors (β3-AR) to produce NO through eNOS activation(Reidenbach et al. 2007), and potentially on β3-ARs located on uterine myocytes, we also tested the effects of LNN-A (100μM, 20 minutes pre-incubation), a potent inhibitor of eNOS activity, ± nebivolol (10 μm), on TNL tissue (n=3 patients per condition, 1-3 myometrial strips per patient), and found that nebivolol did not significantly decrease AUC in the presence of LNN-A (p=0.1405, data not shown), suggesting that nebivolol's action on the myometrium rests primarily on NO production.

To test the effects of combination tocolytics, we first independently dosed TNL tissue (n=3 patients per condition, 1-3 myometrial strips per patient) at the approximate IC_{50} for nebivolol (10 μM) and 18β-GA (100 μM, Fig. 6C). Roughly in line with our experimentally derived IC_{50} data, the AUC for nebivolol dropped to 49.9% (± 6.7) of control ($P<0.0001$), with 18β-GA at 33.8% (± 7.2) ($P<0.0001$), of which there was not a significant difference of AUCs between nebivolol and 18β-GA at their respective IC_{50} values ($P=0.1506$, Fig. 6A). Similarly, the peak tension after nebivolol IC_{50} administration decreased to 72.9% (± 6.5 SEM) of control ($P<0.0001$), an 18β-GA to 33.8% (± 7.2) ($P<0.0001$, Fig. 6B).

When nebivolol and 18β-GA were co-administered for 1 hour (10 μM, 100 μM respectively), their effects on AUC and peak force were additive. AUC dropped to

Fig. 6

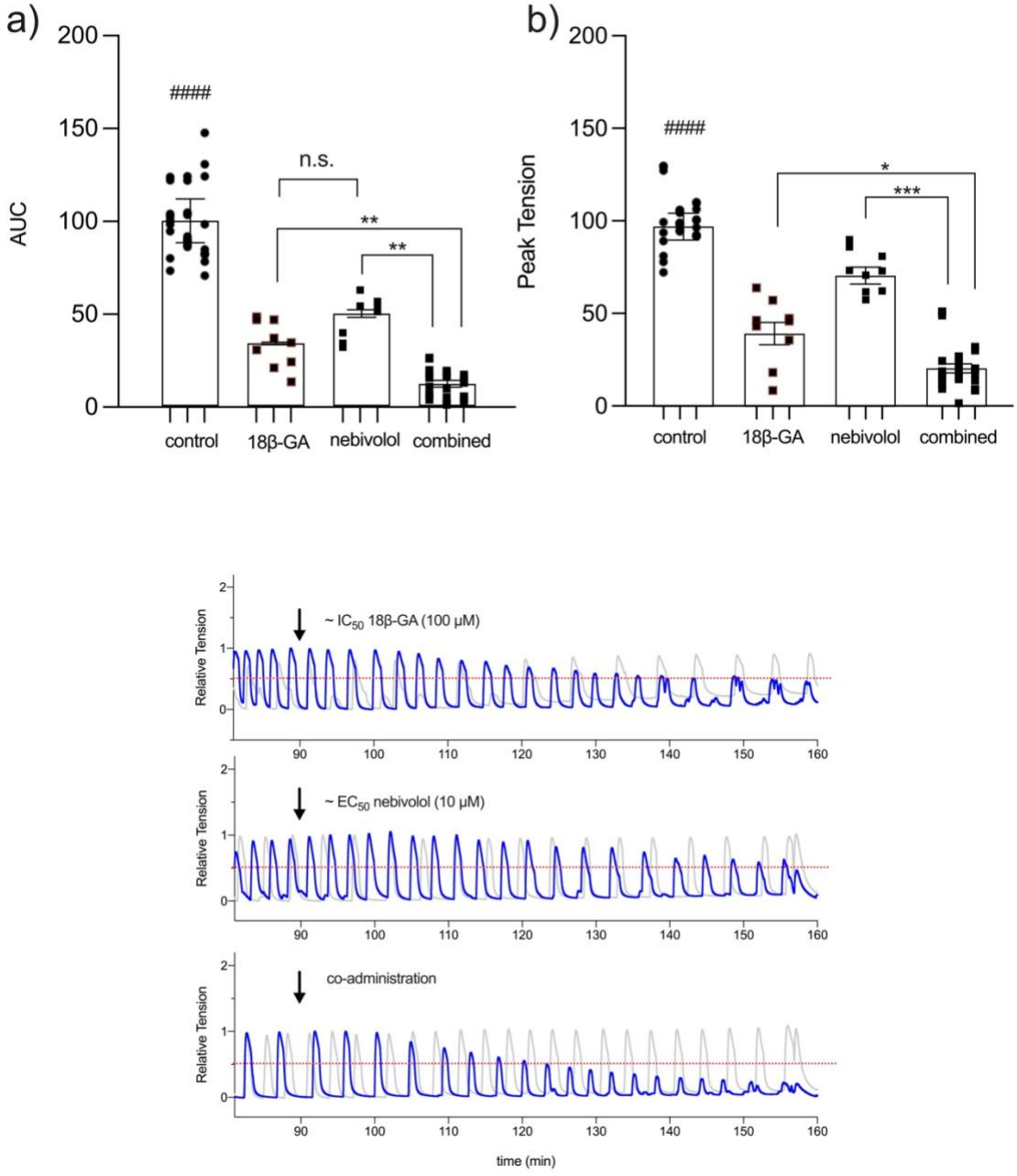

a)
AUC
200
150
100
50
0
####
n.s.
**
**
control
18β-GA
nebivolol
combined
b)
Peak Tension
200
150
100
50
0
####
*
***
control
18β-GA
nebivolol
combined
Relative Tension
~ IC50 18β-GA (100 µM)
~ EC50 nebivolol (10 µM)
co-administration
90
100
110
120
130
140
150
160
time (min)

Figure 6: Coadministration of nebivolol and 18b-glycyrrhetinic acid impart a synergistic negative inotropic effect - TNL myometrium (n = 3 per condition) was hung in an organ bath and exposed to either nebivolol (10 mM), 18b-GA (100 mM), or a combination of both (10, 100 mM). (A) Relative to control there was a significant decrease in AUC for individual and combined doses (P<, 0.0001, t test between control and each dosing scheme), and the coadministration of nebivolol and 18b-GA decreased the AUC to 12.5% (62.5) of control (P<, 0.0001), which represents a 37.4% further decrease over nebivolol alone (P= 0.0061) and an additional 21.3% decrease over 18b-GA alone (P = 0.0088). (B) Similarly, relative to control there was a significant decrease in “peak force” for individual and combined doses (P<, 0.0001, t test between control and each dosing scheme), and the coadministration of nebivolol and 18b-GA decreased peak tension to 21.4% (64.8) over control (P<, 0.0001), which constituted a 51.1% (nebivolol, P = 0.009) and 19.3% (18b-GA, P = 0.0396) drop in peak tension relative to their IC50 doses. (C) Relative change in tension after IC50 administration of “18b-GA” (top panel), “nebivolol” (middle panel), or “both” (bottom panel) in TNL myometrium. Drug dosing represented in blue (—) and control tissue in gray (—). n.s., not significant.

12.5% (± 2.5) of control ($p<0.0001$), which was an additional 37.4% decrease over nebivolol alone ($P=0.0061$), and an additional 21.3% decrease over 18β-GA ($P=0.0088$, Fig. 6A). Co-administration also decreased peak tension to 21.4% (± 4.8) over control ($p<0.0001$), which constituted a 51.1% ($P=0.009$) and 19.3% ($P=0.0396$) drop in peak tension, relative to their IC_{50} values, with nebivolol and 18β-GA, respectively (Fig. 6B).

Src expression during disparate states of pregnancy

Due to the variability of multiple signaling cascades in the myometrium, it is pertinent to understand the relative expression of proteins of interest to determine their relationship to disparate states of pregnancy (Zakar et al. 2020; Barbara M. Sanborn et al. 2005). Total protein lysates from disparate states of human myometrium were run on a western blot to examine Src expression in non-pregnant (NP, n=14), term non-laboring (TNL, n=13), term laboring (TL, n=14), preterm laboring (PTL, n=6), and preterm non-laboring (PTNL, n=6) (Fig. 7) and normalized to GAPDH. Src expression in all states of pregnancy was compared to the NP state. There was no significant difference between Src expression in TNL ($p=0.133$) or PTNL (0.9232) versus NP. There was a significant decrease in in Src expression in TL ($p=0.0072$) and PTL ($p=0.0033$) versus a NP state. When comparing the different laboring states of pregnancy, Src expression is found to decrease in PTL versus TL ($p=0.0031$).

The effect of Src inhibition on mirabegron induced relaxation

Following the derivation of Src expression in disparate states of pregnancy, we next determined its role in β3AR induced relaxation through modulation of MBG

Fig. 7

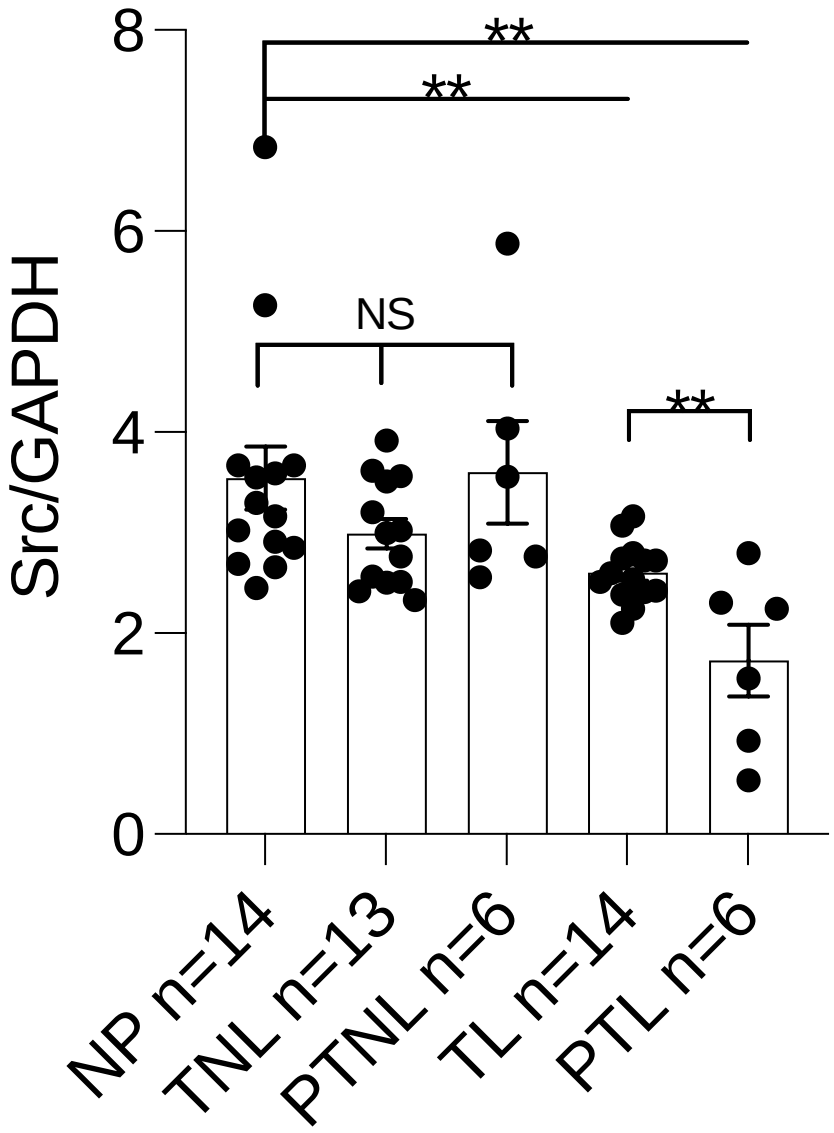


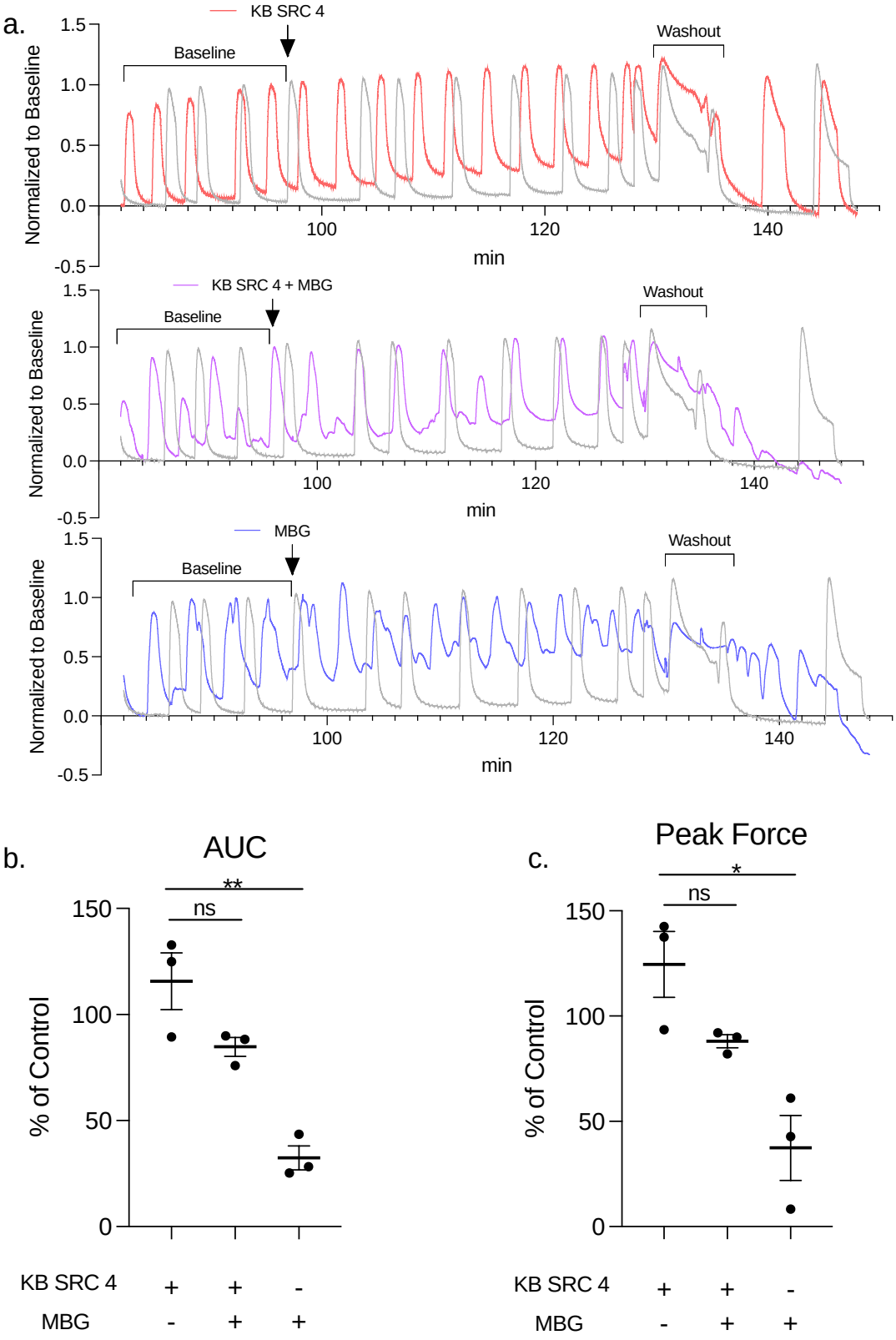
Figure 7: Src expression in disparate states of pregnancy. – When compared to a NP (n=14) state, Src expression does not change in TNL (n=13, p=0.133) and PTNL (n=6, p=0.9232) states of pregnancy. Src expression decreases relative to NP In laboring states of TL (n=14, p=0.0072) and PTL (n=6, p=0.0033). In comparing laboring states only, Src expression decreases more in PTL than in TL (p=0.0031).

treatment. Previous studies have shown that modulation of MBG treatment can determine potential downstream signaling cascades (Asif et al. 2022). Contractile studies were performed on dissected human myometrium tissue strips in oxygenated organ baths, under tension, and treated with 8nM oxytocin to mimic laboring conditions. TNL tissue strips were preincubated with selective Src inhibitor, 30μM KB Src 4, for 15 minutes prior to 30 min 30μM MBG treatment, or treated with 30μM KB Src 4 and 30μM MBG alone. Each tissue strip was normalized to its baseline after oxytocin administration, and to their respective control tissue strip. Each designated 'n' was a unique patient sample consisting of 1-3 strips (Fig. 8). Traces display the relaxation effects of MBG (blue) and a consistent contractile force when treated with KB Src 4 alone (red). A combination of MBG and KB Src 4 decrease the relaxative effects from β3AR activation (purple) (Fig. 8a). Patient samples treated with MBG saw a significant decrease in AUC relative to being treated to KB Src 4 alone (n=3, p=0.0045), while patients treated with MBG and a preincubation period of KB Src 4 saw no decrease in AUC (n=3, p=0.0926) (Fig. 8b). Patient samples treated with MBG also saw a decrease in peak force compared to being treated with KB Src 4 alone (n=3, p=0.0165), while patients preincubated with KB Src 4 and treated with MBG saw no significant decrease (n=3, p=0.0832) (Fig. 8c).

Mirabegron stimulates phosphorylation of Y265 on connexin 43 through Src

Connexin 43 has been shown to be downregulated when phosphorylated on Y265 site (Leithe et al. 2018). With Cx43 being a major potential target for developing novel tocolytic strategies (Barnett et al. 2020), it is

Fig. 8


a.
KB SRC 4
Baseline
Washout
Normalized to Baseline
1.5
1.0
0.5
0.0
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100
120
140
min
KB SRC 4 + MBG
Baseline
Washout
Normalized to Baseline
min
MBG
Baseline
Washout
Normalized to Baseline
min
b.
AUC
**
ns
% of Control
150
100
50
0
KB SRC 4 + + -
MBG - + +
c.
Peak Force
*
ns
% of Control
150
100
50
0
KB SRC 4 + + -
MBG - + +

Figure 8. Src dependent relaxation in the human myometrium with MBG. – a.) Traces show the relative decrease in contractions when treated with 30μM MBG (blue), the partial reduction of the relaxation effects when preincubated with 30μM KB Src 4 (purple), and the stability of contractions with KB Src 4 alone (red) relative to the control (grey). b.) AUC was calculated as a relative percent of the control. 30μM MBG showed a decrease in AUC of contractions (n=3) compared to treatment of 30μM KB Src 4 alone (n=3, p=0.0045). 30μM KB Src 4 + 30μM MBG treatment depicted no difference in AUC of contractions versus 30μM KB Src 4 alone (n=3, p=0.0926). c.) Peak force was calculated relative to the percent of the control. 30μM MBG decreased the peak force of contractions relative to treatment of 30μM KB src 4 alone (p=0.0165). 30μM KB Src 4 + 30μM MBG treatment depicted no difference in AUC of contractions versus 30μM KB Src 4 alone (n=3, p=0.0832)

essential to determine if Cx43 phosphorylation is a targeted downstream consequence of Src activation through β3AR stimulation. phUSMC were plated on 35mm glass bottom dishes, and labeled with phosphorylated Y265 Cx43 antibody (red), Wheat germ agglutinin (WGA) for displaying cellular membrane, and DAPI for nuclear staining. Immunofluorescence (IF) imaging was conducted at 20x to view this relationship between Src and phosphorylated Y265 Cx43 following a one-hour 30μM MBG treatment in phUSMC, with or without a 15-minute prior incubation period of 30μM KB Src 4 (Fig. 9). Imaging revealed an apparent qualitative increase of phosphorylated Y265 Cx43 in MBG treated cells, a decrease when MBG treatment followed a preincubation period of KB Src 4, and little to no expression in control phUSMC.

Downregulation of connexin 43 in phUSMC through mirabegron stimulation

To determine if there is a downregulation of Cx43 as a result of β3AR activation, total Cx43 protein expression in phUSMC was analyzed by western blot following a one-hour 30μM MBG treatment compared to baseline, relative to a control (n=5, Fig. 10a). Following the one-hour 30μM MBG treatment, there was a decrease in total Cx43 expression relative to the baseline MBG treatment ($p=0.0163$); however, this takes into account the new Cx43 being produced at the same time of the treatment period that MBG would theoretically not have an effect on yet.

Previous studies have shown that in order to isolate a specific group of Cx43 within the cells to see how different experimental conditions, such as MBG treatment, can affect their regulation, a pulse-chase experiment can be utilized

Figure 9

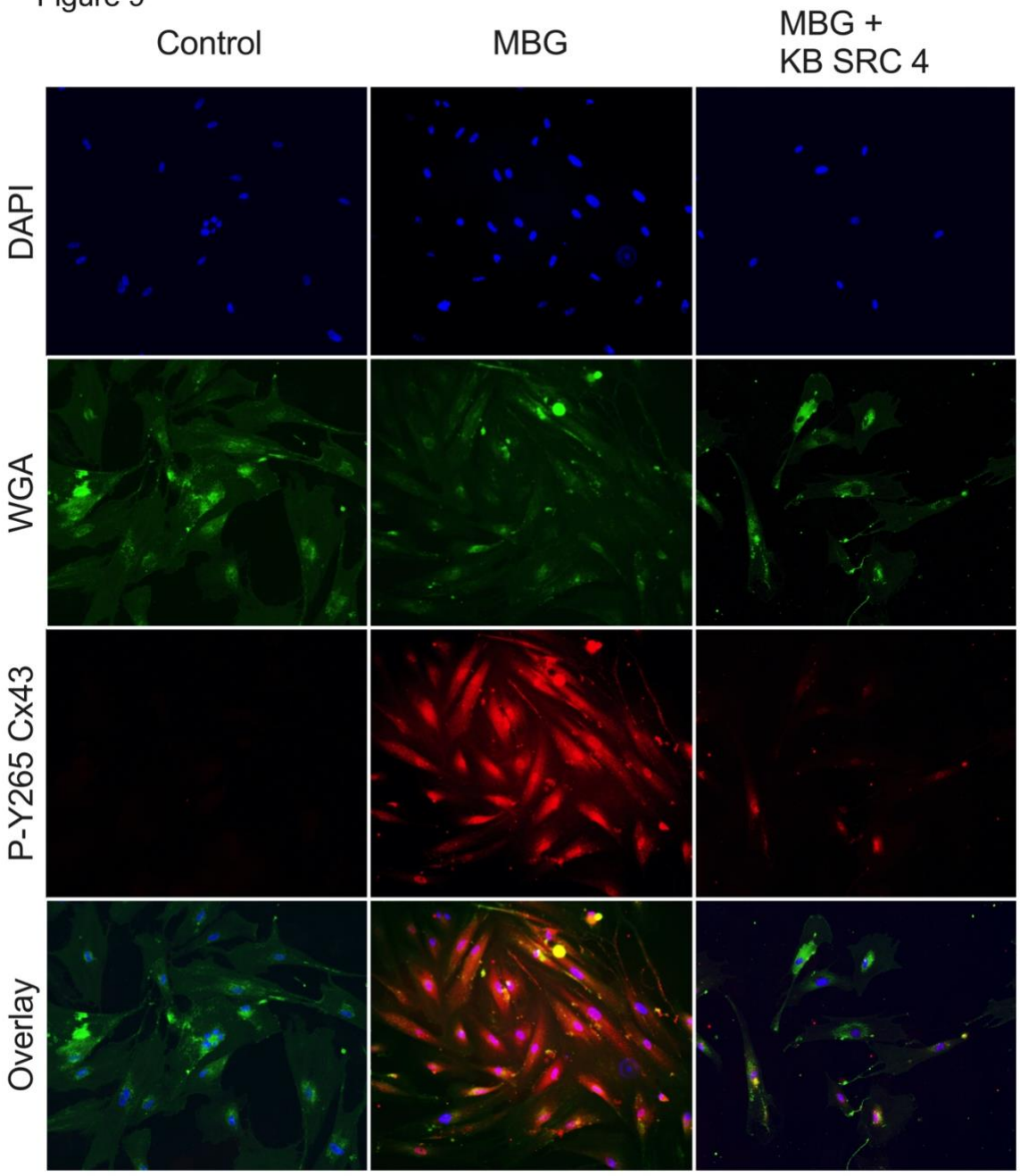


Figure 9. MBG phosphorylation of Y265 Cx43 via Src in phUSMC. – phUSMC were treated with either 30μM MBG, 30μM MBG + 30μM KB Src 4, or volume equivalent of drug solvent for one hour. Immunofluorescent (IF) images (20x) of phUSMC stained with DAPI (nuclear), WGA (membrane), and phosphy-Y265 Cx43 antibodies depict increase in phospo-Y265 Cx43 expression with cells treated with 30μM MBG relative to the control. 30μM KB Src 4 + 30μM MBG decrease the amount of phospho-Y265 Cx43 expression.

Figure 10

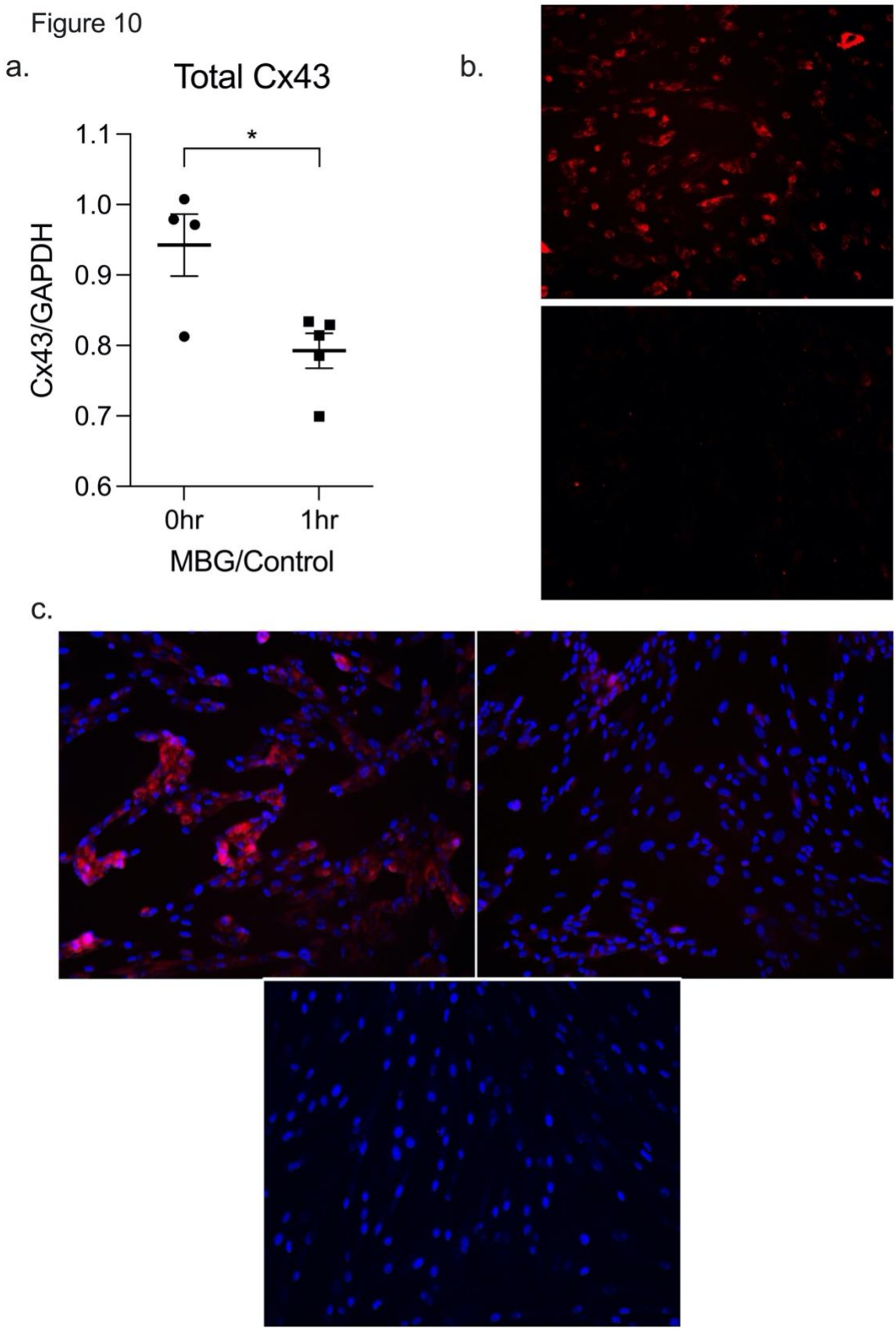


Figure 10. Downregulation of Cx43 expression through MBG treatment in phUSMC. – a.) phUSMC were cultured and grown to full confluency. Cells were treated with 30μM MBG or volume equivalent control for zero hours (n=4) and one hour (n=5). Western blot of resulting cell lysates confirms a decrease in total Cx43 expression after a one-hour dosing period of 30μM MBG relative to the control (p=0.0163) b.) IF images at 10x of transfected GJA1-HaloTag (top) and non-transfected (bottom) phUSMC were incubated with TMR HaloTag ligand to confirm transient transfection of cells. c.) Transfected phUSMC were treated with 30μM MBG (right) or volume equivalent control (left) for 1 hour. Cells were fixed, stained with DAPI, and imaged at 10x. A decrease in Cx43 expression is seen in MBG treated cells. IF images were compared to a negative control (bottom) consisting of non-transfected cells to confirm proper ligand expression.

(Solan and Lampe 2014). By performing a pulse-chase experiment, we can focus primarily on the current Cx43 present in the cell, while disregarding any new Cx43 that may be produced during the treatment period. The phUSMC were transfected with a plasmid containing Cx43 bound to a HaloTag (HT). The transfected phUSMC were dosed with a cell permeable TMR-HT ligand (544ex/570em), followed by a washout so that future Cx43 wouldn't have the bound ligand. Afterwards, IF imaging at 10x was performed to confirm the expression of the ligand in transfected phUSMC (Fig. 10b, top) versus a negative control of non-transfected phUSMC (Fig. 10b, bottom). After confirmation of transfection, phUSMC were treated with 30µM MBG or volume equivalent control for one hour, then fixed and labeled with DAPI. Compared to the control transfected phUSMC (Fig. 10c, left), IF images at 10x of the cells treated with MBG (Fig. 10c, right) displayed a less apparent qualitative expression of the ligand bound to Cx43-HT. Negative control image of non-transfected phUSMC confirmed the absence of non-selective binding of the ligand (Fig. 10c, bottom).

Effect of 24-hour treatment of mirabegron in the human myometrium

Contractile studies were performed in order to evaluate whether the acute quiescent effects of MBG that may affect contractile associated proteins, such as Cx43, can continue to maintain chronic relaxation effects after a 24-hour period. After dissected TNL tissue strips initial dosing of 8nM oxytocin, they were treated with either 30µM MBG or volume equivalent of drug solvent. Each organ bath was placed in a 37^{o}C incubator for 24 hours. Tissues were washed and dosed with 8nM oxytocin to compare the final maximum contractions reached versus the

Figure 11

Post 24hr MBG

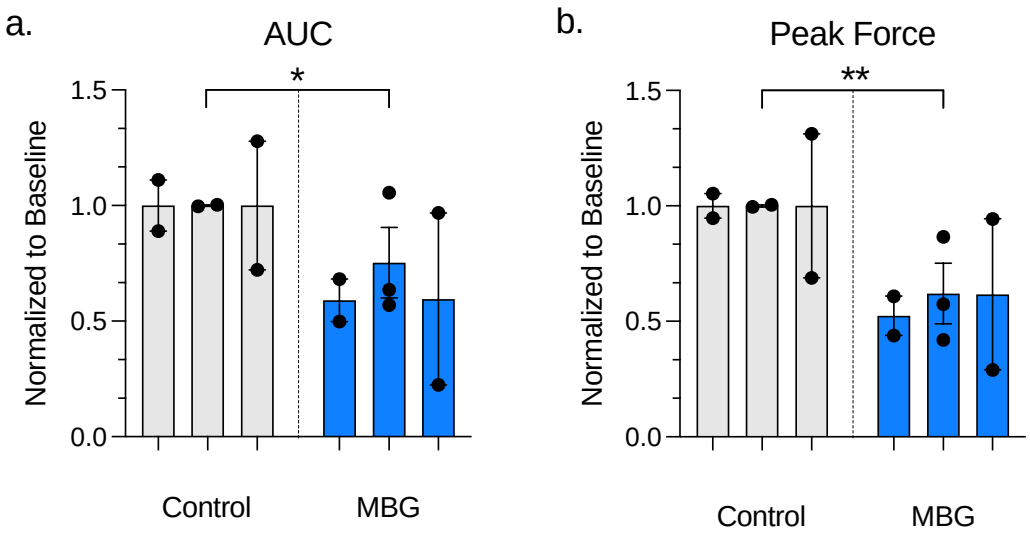


Figure 11. Effects of 24-hour treatment of MBG in the human myometrium. TNL tissue samples (n=3) were treated with 30μM MBG or volume equivalent control for 24 hours. Each tick mark on the x-axis represents a unique patient with multiple tissue strips for each condition. a.) AUC is depicted relative to the baseline for each tissue strip after dosing period. MBG treated tissue samples showed a decrease in AUC over a 24-hour period (p=0.0302). b.) Peak force is depicted as relative to the baseline for each tissue strip. MBG treated tissue samples showed a decrease in peak force over a 24-hour period (p=0.0070).

contractions prior to the 24-hour treatment. Contractions from the tissue strips were normalized to their respective baseline and control tissue strips. The AUC of contractions from MBG treated tissue samples were found to be significantly decreased compared to the AUC of the control tissues after 24 hours (Fig. 11a, n=3, p=0.0302). The peak force of contractions treated from MBG treated tissue samples was also significantly decreased relative to the control tissues after 24 hours (Fig. 11b, n=3, p=0.0070).

Discussion

Preterm birth remains the single greatest cause of neonatal morbidity and hospitalization following pregnancy(Granese et al. 2019; Miniño et al. 2006; Rundell and Panchal 2017). The preterm birth rate in the US and the UK has hovered between 8-12% for decades, resulting in 20,000 infant deaths annually in the US alone(Martin et al. 2013). Novel tocolytic development will benefit from embracing the specific disparities found in women who experience sPTL in lieu of the 'borrowed pharmacology' currently favored. For this reason, we sought to investigate the regulation and modulation of the important myometrial CAP protein, Cx43.

Connexins are ubiquitously expressed in most cell types(Söhl and Willecke 2004). Cx43 is integral to muscle function where its dysregulation can be consequential(Ai et al. 2000; He and Chen 2016). While data surrounding the expression of Cx43, *per se*, does not provide resolution concerning its conformation (GJC vs. HC vs. trafficking), it does afford information regarding the myocyte's state and tissue phenotype. The Cx43 expression data in women is consistent with data from other laboratories(Fig. 1) (Pierce et al. 2002). Of interest, we did not observe differential expression of Cx43 after onset of labor (TNL vs. TL), nor in preterm tissue as compared to term (PTNL vs TNL). Importantly, Cx43 expression was significantly higher in all pregnancy states, relative to NP, with the notable exception sPTL, where Cx43 expression was on par with NP. Similar Cx43 dysregulation in other reproductive tissues during pregnancy affects birth timing; for instance, Cx43 has been found to be downregulated in 1st trimester villi and

decidua of women who experience recurrent pregnancy loss(He and Chen 2016). Further, Wnt-1 activation is known to increase expression of Cx43 in myocytes(Ai et al. 2000), and it has been shown that *WNT1* is downregulated in the fetal membrane of women who experience PTL(Pereyra et al. 2018). As such, our data further correlates Cx43 dysregulation to aberrant labor, and due to Cx43's integral role as a smooth muscle CAP protein, this dysregulation suggest that Cx43 may be a unique target of interest for tocolytic development.

Beyond Cx43 protein expression, the posttranslational state of Cx43 is fundamental to its function. Phosphorylation of S368 is of keen interest as this posttranslational modification decreases GJC conductance(Lampe et al. 2000) and facilitates its internalization(Boswell-casteel et al. 2016; Ribeiro-Rodrigues et al. 2017; Ek-Vitorín, Pontifex, and Burt 2018). Our finding that NO increases the ratio of pS368 in myometrium (Figure 2A) is significant in that it bolsters a growing body of evidence that confirms our previous data demonstrating NO-mediated cGMP-independent relaxation of the human myometrium(K K Bradley et al. 1998). In addition to altering its phosphorylation state, we also determined that that Cx43 is directly S-nitrosated by NO during relaxation of the myometrium (Figure 2B), which has been shown to alter conformation and conductance of the channel(Dimitrova et al. 2017; Straub et al. 2011; Retamal et al. 2006). Together, these data provide compelling evidence that NO's action on Cx43 serves as an important mediator of relaxation in the myometrium.

Based on our results, we elected to investigate the effect of NO on HC function. The HC is associated with quiescence in that it permeates PGE_2(Burra

and Jiang 2009) and other small molecular weight mediators, including ATP(King and Lampe 2005), into the extracellular space. Cx43-SNO-C271 has been shown to activate both the HC(Contreras et al. 2003) and the GJC(Straub et al. 2011) at the myoendothelial junction. In line with these finding, our data indicate that NO activates the HC in human uterine smooth muscle cells under physiological extracellular Ca^{2+}, and this activation is blocked with the addition of an HC-selective inhibitor, TAT-Gap19 (Figure 3). Our finding that the HC open-state is correlated to the presence of NO provides additional data in support of the non-canonical relaxation of the myometrium by NO. Whether HC activation is critically mediated by SNO-Cx43 formation, or pS368-Cx43 formation, or both, awaits future investigation.

The effect of NO on the biochemical state of Cx43 in the myometrium is compelling; however, simply modulating Cx43's SNO or O-phosphorylation state does not guarantee tocolysis in myometrial tissue. For this reason, we sought to determine whether pharmacological mediation of Cx43 and NO exhibits novel tocolytic properties. To this end, we first independently overserved the effects HC and GJC inhibition on myometrial contractile dynamics. As we hypothesized, the inhibition of the HC with TAT-Gap19 conveyed positive inotropic effect on the myometrium (Figure 4), further reinforcing the concept that the HC is critical to the maintenance of myometrial quiescence. Conversely, the inhibition of Cx43 with 18β-GA, which also promotes Cx43-pS368 formation(Liang et al. 2008), blunted contractile dynamics (AUC) in a dose-depend manner (Figure 5B). Taken together,

these data indicate that activation of the HC and inhibition of GJC may function in concert to mediate quiescence.

With the therapeutic potential of GJC inhibition established, we posited that increasing endogenous NO-availability would further enhance tocolysis by promoting Cx43 S-nitrosation and Serine O-phosphorylation (pS368) to increase HC activity and blunt that of the GJC. We have previously demonstrated, in an limited manner, that NO generation through eNOS activation (nebivolol, Bystolic™, Allergan)(Scott D. Barnett and Buxton 2018) exhibits a tocolytic effect. Here we expanded upon that finding and determined that nebivolol conveys robust negative inotropic effects on the myometrium in a dose-dependent manner (Figure 5). Interestingly, because nebivolol has some agonism activty of β3ARs, we must consider some of the relaxation effects stemming from β3AR activation. We elected to co-administer 18β-GA and nebivolol at their IC_{50} values (Figs. 5 and 6) and found that the combined effect on AUC and peak tension was synergistic, all but abolishing contractions. Further studies must be conducted to determine if the tocolytic properties of 18β-GA and nebivolol fully transfer to PTL and to directly correlate β3AR activation to S-nitrosation events on Cx43.

Although nebivolol has some agonistic activity towards β3AR, it is highly selective for antagonizing β1 adrenergic receptor (Fongemie and Felix-Getzik 2015). Thus, in order to understand mechanisms surrounding β3AR and Cx43, we used the selective β3AR agonist, MBG.

The β3AR has been shown to have unique characteristics compared to its β1 and β2 counterparts to make it a potential target for tocolytic strategies. Some

of these characteristics include being overly expressed in the myometrium during pregnancy, lacking the binding motif necessary to be downregulated by β-arrestin, and being able to relax contracting human myometrium tissue (Asif, Barnett, and Buxton 2022; Schena and Caplan 2019). Previous studies have shown that the β3AR promotes relaxation through pathways involving different downstream signaling cascades (Asif, Barnett, and Buxton 2022). This study focuses on the relationship of Src and contractile associated protein Cx43 following β3AR stimulation.

As the myometrium prepares to go in labor it upregulates and downregulates the necessary pathways to promote a laboring state (Cappelletti et al. 2016; Mor et al. 2011). Src is a multipurpose kinase with multiple downstream targets depending on its upstream signaling (Haura 2006). Src has been shown to be involved in relaxation through inhibition of reactive oxygen species in macrophages (Hadi et al. 2017), and in contractile associated processes involving focal adhesion signaling (Morgan 2014). We show that Src protein expression is significantly decreased in laboring states of pregnancy compared to non-pregnant and non-laboring states (Fig. 7). Src protein expression is also decreased in PTL to a greater extent than TL (Fig. 1). The myometrium upregulates and downregulates different proteins in order to transition to a state of labor (Salomonis et al. 2005). The downregulation of Src in labor supports the notion of it being more so involved in smooth muscle relaxation mediated pathways.

Although part of the β3AR interactions to Src has been previously studied, the effect on contractile studies on human myometrium tissue involving the two

have yet to be identified (Hadi et al. 2013; 2017). To determine this relationship of Src and β3AR mediated relaxation, we treated human myometrium tissue with both MBG and a selective Src inhibitor. We determined that inhibition of Src decreases the quiescent effects of MBG (Fig. 8). This emphasizes the importance of Src in mediating relaxation through the β3AR pathway.

Cx43 gap junctions are involved with intercellular communication to promote calcium exchange and propagate contractions, thus making it an attractive target for inhibition (Kidder and Winterhager 2015; Scott D. Barnett, Asif, Anderson, et al. 2020). Previous studies have shown that Src-mediated phosphorylation of Cx43 on Y247/Y265 downregulate Cx43 (Solan and Lampe 2020), which lead us to investigate whether the β3AR can induce the downregulation as well. Through IF imaging we show that β3AR activation phosphorylates Y265 Cx43 through a Src dependent manner (Fig. 9). Also, we show that total Cx43 protein expression is downregulated after treated with β3AR agonist (Fig. 10a). In order to visualize the effect of β3AR on Cx43 while excluding the new Cx43 being formed, a pulse-chase experiment was utilized on primary cells transfected with Cx-43 HT. IF imaging depicted the decrease in Cx43 from β3AR activation (Fig. 10c). β adrenergic receptors were previously found to enhance Cx43 gap junctions in cardiomyocytes (Yi Zhang et al. 2020); however, in the myometrium smooth muscle we show that β3AR activation promotes the downregulation of Cx43.

Although the acute effects of β3AR activation on human myometrium tissue have been determined, the association with decreasing the intercellular

communication through downregulating Cx43 lead us to determine whether the β3AR can have long lasting quiescent effects. After a 24-hour treatment with MBG, we show that human myometrium tissue still has a decrease in its contractile properties against control tissues (Fig. 11). This suggests that β3AR can have potential for long term treatment strategies.

While the solution to the problem that is PTL continues to elude the scientific community, there is little doubt that to decipher this mystery we must better understand dysregulated pathways during affected pregnancies, and in turn develop novel therapeutics that mitigate these disparities. Cx43 is a foundational CAP protein in the myometrium, and here we show that it is dysregulated in sPTL myometrium and its function is moderated by NO. By inhibiting GJC activity, and increasing endogenous NO-availability, we were able to severely blunt contractions, reinforcing the concept that pharmacologic intervention of multiple dysregulated pathways during pregnancy enhances quiescence. The association between β3AR and the downregulation of Cx43 has not been shown before in the myometrium. This downregulation of a contractile associated protein is now added to the list of mechanisms of relaxation that β3AR stimulates, including NO production and BK_{Ca} activation. Further studies are still required to determine if increasing endogenous NO from β3AR activation can increase S-nitrosation of Cx43. Nonetheless, β3AR agonist, MBG, continues to be a appealing target in the development of tocolytic strategies for PTL, and leads us to inquire if combinational tocolytic strategies involving Cx43 GJ inhibitor would have greaer desired effects.

Chapter 4

Novel Identification and Modulation of the Mechanosensitive Piezo1 Channel in Human Myometrium

Disclosure Statement

The authors declare they have no conflict of interest. Permission granted to use published work for dissertation (Scott D. Barnett, Asif, and Buxton 2022). (Appendix A)

Abstract

Approximately 10% of US births deliver preterm before 37 weeks of completed gestation. Premature infants are at risk for life-long debilitating morbidities and death, and spontaneous preterm labor explains 50% of preterm births. In all cases existing treatments are ineffective, and none are FDA approved. The mechanisms that initiate preterm labor are not well understood but may result from dysfunctional regulation of quiescence mechanisms. Human pregnancy is accompanied by large increases in blood flow, and the uterus must enlarge by orders of magnitude to accommodate the growing fetus. This mechanical strain suggests that stretch-activated channels may constitute a mechanism to explain gestational quiescence. Here we identify for the first time that Piezo1, a mechanosensitive cation channel, is present in the uterine smooth muscle and microvascular endothelium of pregnant myometrium. Piezo is downregulated during preterm labor, and stimulation of myometrial Piezo1 in an organ bath with the agonist, Yoda1, relaxes the tissue in a dose-dependent fashion. Further, stimulation of Piezo1 while inhibiting PKA, AKT, or eNOS mutes the negative inotropic effects of Piezo1 activation, intimating that actions on the myocyte and endothelial nitric oxide signaling contributes to Piezo1-mediated contractile dynamics. Taken together, these data highlight the importance of stretch-activated channels in pregnancy maintenance and parturition, and identify Piezo1 as a tocolytic target of interest.

Introduction

Preterm labor (PTL) and preterm birth (PTB) have plagued society since time immemorial. PTB is the leading cause of death in children under 5 (Chawanpaiboon et al. 2019), and those who survive commonly suffer from life-long deleterious health disparities (Cooke 2006). PTL, defined as sustained contractions prior to 37 weeks of gestation, occurs in about 10% of all pregnancies (Martin et al. 2021), with women of African descent being as much as 40% more likely to deliver preterm (Korinek and Ahmmad 2021), and their infants twice as likely to die as a result (Burris and Parker 2021). While advances in perinatal care have ameliorated many serious complications if addressed during late preterm (> 32 weeks), each year thirteen million infants are born preterm globally, costing in excess of 38 billion annually (adjusted) in the Unites States alone (Outcomes and Press 2007). While there are many known correlates to PTL/PTB, such as infection, smoking/drug use, and even race, about half of all PTL is idiopathic, also known as spontaneous preterm labor. After nearly 70 years of active tocolytic development (Abramson and Reid 1955) there are still no FDA-approved drugs that can significantly delay PTB beyond 48-hours allowing afflicted pregnancies to go to term. Our research seeks to explore distinctive/dysregulated pathways of the uterus to identify novel targets for tocolytic development. Here we investigate Piezo1 (*PIEZO1*), a stretch-activated cation channel (SAC), to better understand myometrial contractile dynamics.

The myometrium is the smooth muscle of the uterus which contracts to expel the infant during labor. Little is known about the cytoarchitecture of human myometrium in pregnancy (Sweeney et al. 2014). The myometrium is over 90% muscle cells by volume, and the cellular heterogeneity includes blood vessels, fibroblasts, immune and stem cells (Santamaria et al. 2018). Importantly, unlike gastrointestinal smooth muscle that employs both nervous innervations and pacemaker cells (Kenton M Sanders et al. 2016; K M Sanders and Smith 1986), human myometrial muscle fibers are interwoven, do not form distinct layers (Blanks, Shmygol, and Thornton 2007); are not innervated by motor nerves (Tingåker and Irestedt 2010); and a pacemaker cell type has not been convincingly described (Roger Charles Young 2018; Susan Wray and Prendergast 2019). These peculiarities of myometrium, combined with the finding that uterine myocytes preferentially relax to nitric oxide via protein S-nitrosation (Scott D. Barnett et al. 2018), and not cyclic nucleotide generation (K K Bradley et al. 1998; Lai, Tribe, and Johnson 2016), has led us to investigate quiescent-mediated pathways specific to the myometrium.

Wholly unique in human physiology is the demand placed on the uterus to remain quiescent for the entire 40 weeks of gestation. No other muscle must remain functionally dormant for such an extended period. While the pathways that ensure this prolonged quiescence are largely unknown, a logical approach is to investigate actions and stresses unique to the uterus during pregnancy. As such, investigating SACs in the myometrium during gestation is not only reasonable, but may unearth the underlying pathophysiology of PTL. There are several

mechanosensitive channels in the myometrium, including the inward rectifying Ca^{2+} channel, TRPV4 (*OTRPC*) (Villegas et al. 2021), and the outward rectifying K^{+} channel, TREK-1 (*KCNK2*) (Heyman et al. 2013; Buxton et al. 2011), both known to be regulators of membrane polarization during pregnancy. Due to the extraordinary hydrostatic load imposed on the uterus as the pregnancy progresses, here we seek to identify and examine the SAC Piezo1, to determine its contribution to pregnancy maintenance and labor.

Piezo1 ('piesi' meaning pressure in Greek) is a mechanosensitive inward rectifying cation channel (Coste et al. 2012) which is preferential to Ca^{2+} under physiologic conditions (Romac et al. 2018). Piezo1 was first identified in astrocytes in the mid 2000s (Satoh et al. 2006), and is the subject of the 2021 Nobel Prize in Physiology or Medicine. It has since been found in other tissues (J. Li et al. 2014; John et al. 2018), including human endometrium (Hennes et al. 2019), where its aberrant expression is thought to contribute to preeclampsia (Arishe, Ebeigbe, and Webb 2020). Importantly, until now Piezo1 has not been characterized in human myometrium. Piezo1 assembles in the membrane as a large trimer of ~286 kD (Coste *et al.*, 2010; Gottlieb & Sachs, 2012), with a single channel conductance of ~37 pS (Gottlieb, Bae, and Sachs 2012). It is selectively agonized/antagonized by the small molecules Yoda1 and Dooku1, respectively (Botello-Smith et al. 2019; Evans et al. 2018; Lhomme et al. 2019), which allows for precise experimental modulation of the channel. Due to its mechanosensitive property and permeability to Ca^{2+}, Piezo1 is an attractive protein of interest for investigating pregnancy maintenance.

Of all divalent cations, Ca^{2+} is the preeminent modulator of smooth muscle activity. Ca^{2+} is a critical agonist and second messenger in both myocytes and microvascular endothelial cells (MECs) of the myometrium. In the myocyte Ca^{2+} is most notably recognized as an initiator of contraction via calmodulin-mediated myosin light chain kinase activation, but it also activates membrane bound BK_{Ca} channels (Maxi-K, *KCNMA1*), driving K^+ efflux (Nardi and Olesen 2008). Piezo1 activation in human myometrium following TRPV4 stimulation has been found to activate BK_{Ca}, resulting in relaxation of the tissue (Villegas et al. 2021), while in human arterial fibroblasts an association was found between Piezo1 stimulation and BK_{Ca} activity (Jakob et al. 2021). In vascular smooth muscle BK_{Ca} is part of a localized signaling complex that includes the L-type calcium channel, the sarcoplasmic reticulum, and TRPV4, driving polarization of the membrane (Dopico, Bukiya, and Jaggar 2018). MECs, on the other hand, which directly interface with the myocytes of the myometrium, are sensitive to mechanical stimuli generated by blood flow (pulsatile stretch and shear stress) within the expanding uterus. These stimuli trigger a physiological response *via* release of vasodilatory factors such as nucleotides (Buxton et al. 2001; Wang et al. 2016) and nitric oxide (Vanhoutte et al. 2017), which are mediated in part by stimulation of protein kinase A (PKA) (Bir et al. 2012) and AKT, sometime called protein kinase B (X. P. Zhang and Hintze 2006). As such, Ca^{2+} entry into uterine myocytes and MECs via Piezo1 may act as an important quiescent regulator.

Uterine myocytes and MECs must endure substantial mechanical stress during pregnancy and have evolved to leverage the functionality of SACs to regulate

homeostatic function. We have elected to explore Piezo1 expression and function in these cell types, and in whole myometrial tissue, to determine if Piezo1 significantly modulates their function. We posit that Ca^{2+} influx via Piezo1 in myometrial MECs stimulates nitric oxide production via AKT/PKA activation, promoting quiescence, and that Piezo1 in pregnant human uterine smooth muscle (phUSMC) contributes to quiescence, in part, through Ca^{2+} mediated BK_{Ca} activation.

Materials and Methods

Ethical Approval: All human tissue collection was obtained in accordance with the Declaration of Helsinki and approved by the Institutional Review Board at the University of Nevada Biomedical Review Committee for the protection of human subjects (approval 509108-19). All experiments were performed in accordance with the NIH guidance on the use of human tissues in research.

Tissue collection: Human uterine biopsies were obtained with written informed-consent from mothers with singleton pregnancies undergoing Cesarean section as previously described (Scott D. Barnett, Asif, Anderson, et al. 2020). Exclusion criteria in pregnant women include: uterine or generalized infection to include COVID-19, a maternal age < 18 years, any history of drug abuse, co-morbid diagnoses such as HIV infection or AIDS, hepatitis C infection, uncontrolled diabetes, renal disease, and any use of steroids other than betamethasone (including topical use) during pregnancy. Tissues were transported to the laboratory immediately in cold Krebs buffer containing, 118 mM NaCl, 4.75 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO4, 25 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 20 mM dextrose, and adjusted to pH 7.4. Tissues were dissected under 4x magnification to isolate smooth muscle, then either immediately employed in contractile experiments or snap frozen in liquid nitrogen and stored in a vapor-phase freezer unit at -150 °C.

Western Blot: Total protein was collected from whole tissue and cell culture. In all cases protein was isolated in MAPK buffer containing: 60 mM Tris-HCL (pH 6.8), 1% glycerol, 2% SDS, 1 µM leupeptin, 1 mM EGTA, 1 mM EDTA, 1 mM Na_3VO_4, and protease/phosphatase inhibitors (PPC110: Sigma Aldrich, St. Louis, MO). Tissue samples were frozen and crushed using a liquid nitrogen-cooled mortar and pestle, followed by wet homogenization (gentleMACS™ Dissociator, Miltenyi Biotec Inc., North Rhine-Westphalia, Germany). Cultured cell lysates were collected mechanically (cell scraper) using the buffer described above after (3) washes in sterile PBS. Sample concentrations were determined using EZQ (R33200: Thermo Fischer, Waltham, MA).

Polyacrylamide Gel electrophoresis (PAGE) - For all samples 60 µg of total protein lysate was separated on a 4-15% polyacrylamide gel at 160 V for ~60 minutes, then transferred to a polyvinylidene fluoride (PVDF) membrane and blocked in 5% blotting-grade nonfat milk/TBST (Biorad, 1706404, Hercules, CA) overnight. Western blots were labeled with mouse monoclonal anti-Piezo1 1° antibody (1:500, MA5-32876; Thermo Fischer, Waltham, MA) followed by Goat anti-Mouse IgG (H+L) Cross-Adsorbed 2°, HRP (1:5000, Cat. A16072, Thermo Fischer) with SuperSignal™ West Pico PLUS activator (Cat. 34580, Thermo Fischer). GAPDH was used as a control protein and blots were labeled with anti-GAPDH 1° antibody (1:1000, Cat. sc-47724, Santa Cruz Biotechnology) followed by Goat anti-Rabbit IgG (H+L) 2° Antibody, HRP (1:5000, Cat. 31460; Thermo Fischer). Blots were stripped between each antibody application (Cat. 928-40030,

LI-COR Biotechnology, Lincoln, NE). Each data point was from a unique patient and was treated as an individual ‘n.’

Cell culture: 1° cells were generated from TNL human myometrium as previously described (Asif, Barnett, and Buxton). Cells were detached from flasks using a collagenase (CLS2, Worthington, US) and trypsin (27250-018, Gibco, US) enzyme solution (2:1 collaganse:trypsin) in MACS buffer and Gibco Dulbecco’s Modified Eagle Medium (DMEM, 11995-065, Gibco, Waltham, MA). Using a gentleMACS™ Dissociator, cells were agitated 3x for 90 seconds with 45-minute rest at 37°C between agitations. The digestion was triturated 3x and filtered through a 100 µM sterile mesh. Cells were the cultured to 80% confluency, preincubated with FcR blocking reagent, then separated over CD31+ bead LS columns using a MidiMACS separator (Miltenyi Biotec:130-091-935, Auburn, CA). Cells captured by the beads were deemed CD31+ pregnant human myometrial endothelial cells (phMEC) and CD31- pregnant human uterine smooth muscle cells (phUSMC). phMECs cultured in endothelial basal medium 2 (C-22011, PromoCell, Heidelberg, Germany) containing 10% FBS and 1% penicillin, while the phUSMC cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 50 U/ml streptomycin, 50 µg/ml penicillin, and 10% FBS and supplemented with estrogen (15 ng/ml) and progesterone (200 ng/ml). All cells were cultured in a balanced oxygen (95%:5% O_2:CO_2) incubator at 37°C. Piezo1-deficient HEK293T cells ($Piezo1^{KO}$) (Lukacs et al. 2015) were kindly provided by Dr. Ardem Patapoutian of the Howard Hughes Medical Institute at Scripps Research and were cultured in DMEM with 10% FBS (without antibiotics).

Immunofluorescence: phMEC (CD31+), phUSMC (CD31-), and Piezo1^{KO} cells were plated on 35mm glass bottom dishes (MatTek Corporation) and grown to ~80% confluence. Prepared cells were treated for 15 minutes with 4% paraformaldehyde, followed by 0.5% triton-x for 5 minutes and 5% BSA blocking buffer for 1 hour with 3x PBS washes between each step. Cells were then labeled with Piezo1 monoclonal 1° antibody (1:100, MA5-32876, Thermo Fisher Scientific), followed by 2° Alexa Fluor® 594 (1:100, ab150080, Abcam) and imaged at 10x magnification on an inverted fluorescent microscope (ECHO, San Diego, CA). All cells were additionally labeled with wheat germ agglutinin (WGA) conjugated to Alexa Fluor® 488 (1:00, W11261, Thermo Fisher Scientific), followed by DAPI mounting medium (H-1500, Vector Laboratories, Burlingame, CA). Exposure and contrast were adjusted globally to ensure adequate visibility of each channel. All images were taken with negative controls (absence of primary antibody) to verify the absence of nonselective secondary binding (data not shown).

Calbryte™ Ca^{2+} permeability assay: 1° cultured (p2-p4) phUSMC, phMEC, and Piezo1^{KO} cells were seeded to a density of 4000 cells/well in a half-volume flat bottom 96-well microplate (675076, Greiner Bio One, Kremsmünster, Austria) and left to settle for 24 hours. Just prior to start of experiment media was replaced with Ca^{2+}-free KREBS containing 20 mM HEPES ($buffer_{exp}$) and cells were incubated for 10 minutes in either 10 nM oxytocin (phUSMC) (Gimpl and Fahrenholz 2001) or 10 mM caffeine (phMEC/Piezo1^{KO}) (Corda et al. 1995) to deplete the sarcoplasmic/endoplasmic reticulum of Ca^{2+}. Following incubation, 200 nM thapsigargin (Xuan, Wang, and Whorton 1992; Wictome et al. 1992) was added

for an additional 15 minutes to prevent Ca^{2+} re-uptake. Cells were then rinsed 2x in $buffer_{exp}$ containing 0.04% Pluronic® F-127 and 1 mM probenecid (anion transporter inhibitor), followed by 5 µM Calbryte™ 520 AM (Cat. 20650 - AAT Bioquest, Sunnyvale, CA) for 1 hour. Following incubation period cells were rinsed 2x in $buffer_{exp}$ containing 0.04% Pluronic® F-127 and 1 mM probenecid, followed by addition of 100 nM amlodipine (Ca^{2+} channel blocker), Yoda1 ± Dooku1, and 2.5 mM Ca^{2+}. Negative controls were run in either the absence of Calbryte™ 520 AM, or the absence of extracellular Ca^{2+} (data not shown). Each experimental condition was run 18 times over three plates, with six replicates per condition, per plate. For analysis, ΔCa_i^{2+} was calculated as follows: (1) value of blank cell (all reagents minus Calbryte) was subtracted from value of experimental cell; (2) resultant value normalized to the A280 for each condition to account for variations in the number of cells loaded into each well; (3) resultant value subtracted from the average value at t=0 for each condition to provide the 'delta,' or change in fluorescence over the experimental period.

Contractile studies: In a temperature controlled (37°C) organ bath (DMT 820MS, Danish Myo Technology, Hinnerup, Denmark) containing oxygenated (95%:5% O_2:CO_2) Krebs buffer(Scott D. Barnett et al. 2018) strips of myometrium (~0.5 × 15 mm) from the superior portion of the transverse incision were clip-mounted to a force transducer and stretched isometrically to L_0 then held at 2 grams of final tension. The relationship between experimental stretch to achieve L_0 and its impact on the activity of stretch-activated Piezo1 was tested in a set of experiments where tissues were stretched to 1 rather than 2 grams. Lowering the

initial tension had no effect on the ability of the Piezo1 agonist Yoda1 to activate the channel. Tissues were challenged with KCL (60 mM replacing NaCl) for 3 minutes, followed by wash-out, then allowed to equilibrate for 1 hour, or until regular spontaneous contractions were observed. Only tissues that responded to KCL-challenge were employed in experiments. Tissues were further challenged with 8 nM oxytocin to mimic endogenous laboring conditions. Control tissues were exposed to a volumetric equivalent of drug solvent. Tissues were pretreated for 15 minutes with the either the PKA inhibitor, 'PKI 14-22 amide, myristoylated' (*myr*PKI_{14-22} 10 µM, Cat. No. 2546 - Tocris, Minneapolis, MN) (Harris, Persaud, and Jones 1997), the AKT inhibitor FPA-124 (10 µM, Cat. No. 2926 - Tocris, Minneapolis, MN) (Strittmatter et al. 2012) the eNOS inhibitor N_{ω}-Nitro-L-arginine, (L-NNA 100 µM - N5501 MilliporeSigma, St. Louis, MO), the *BK_{Ca} inhibitor, Paxilline (10 µM, Cat. No. 2006, Tocris), or* the *BK_{Ca} activator, NS1619 (30 µM, Cat. No. 3804, Tocris),* ± 3 µM Yoda1 (~EC_{50}) for 60 minutes. Area under the curve (AUC), peak tension, and contractile frequency were analyzed using LabChart (version 8.1.12, Win10, ADInstruments., Colorado Springs, CO).

Statistical Analysis: For organ bath experiments each 'n' is a unique patient from which the values of 1-3 myometrial strips for each condition were averaged. The last three contractions of each dosing and control period were analyzed to determine AUC and peak tension. Peak tension is defined as the maximum tension at the height of contraction and subtracted from the minimum tension for each given contraction. Each tissue strip was normalized to its own baseline prior to dosing, then to the average of all control strips to account for rundown (controls

are vol. equivalents of drug solvent; DMSO for Yoda1, Dooku1, FPA-124, Paxilline, NS1619 and KREBS for *myr*PKI_{14-22} and L-NNA). The average of all control strips for each 'n' was set to a nominal value of '100' so that data is presented as a "% change from control." All tissues that did not recover (regain contractions) following washout were rejected for analysis. Contractile frequency is defined the as the number of contractions in the final 15 minutes of the dosing interval divided by the number of contractions during the 15 minutes period just prior to dosing and presented as percentage.

For all experiments, Student's t-test were unpaired and two-tailed. Normally distributed data were analyzed with a Welch's correction to account for variable SDs, while non-normal data were subjected to a Mann-Whitney test. Likewise, either an ordinary one-way ANOVA test was employed, or a Kruskal-Wallis test for non-parametric data, as appropriate. All error bars on graphs displayed as SD unless otherwise stated. All data were analyzed using Prism (v. 9.3.1, Graphpad Software, San Diego, CA).

Results

Expression of Piezo1 during pregnancy

Total protein from full-thickness human myometrium was run on a Western blot (n=5 per sample type). Samples were collected during the following states of pregnancy and labor: non-pregnant (NP), preterm non-laboring (PTNL), term non-laboring (TNL), preterm laboring (PTL) and term laboring (TL). Blots were probed for Piezo1 and normalized to GAPDH, with the average of all NP values set to a nominal value of '1.' Analysis determined that Piezo1 is most highly expressed in TL tissue (Fig. 1), with a 3.57-fold increase in expression over NP (P=0.0117). Piezo1 is also more significantly expressed in TL vs. PTNL (P=0.0427), TL vs. TNL (P=0.0126), PTNL vs. PTL (P=0.0020) and most notably TL vs. PTL (P=0.0028), with a 13.91-fold difference in Piezo1 expression. There was no significant difference in expression of Piezo1 between NP/TNL/PTNL (ANOVA, P=0.1033). Patient demographics and relevant pregnancy data (Table I) are provided in recognition that health disparities are known correlatives to PTL (de Oliveira et al. 2018), and because better systematic preterm reporting metrics are needed (Chawanpaiboon et al. 2019).

Immunofluorescent imaging and quantification of Piezo1 in myometrial CD31+ and CD31- cells

To determine distribution of Piezo1 in the two major myometrial cell types, CD31+ (phMEC) and CD31- (phUSMC), immunofluorescence and Western blots were used. *Immunofluorescent imaging*: Primary cells were plated on 35 mm glass bottom dishes, then labeled with the nuclear stain, 4',6-diamidino-2-phenylindole,

Fig.1

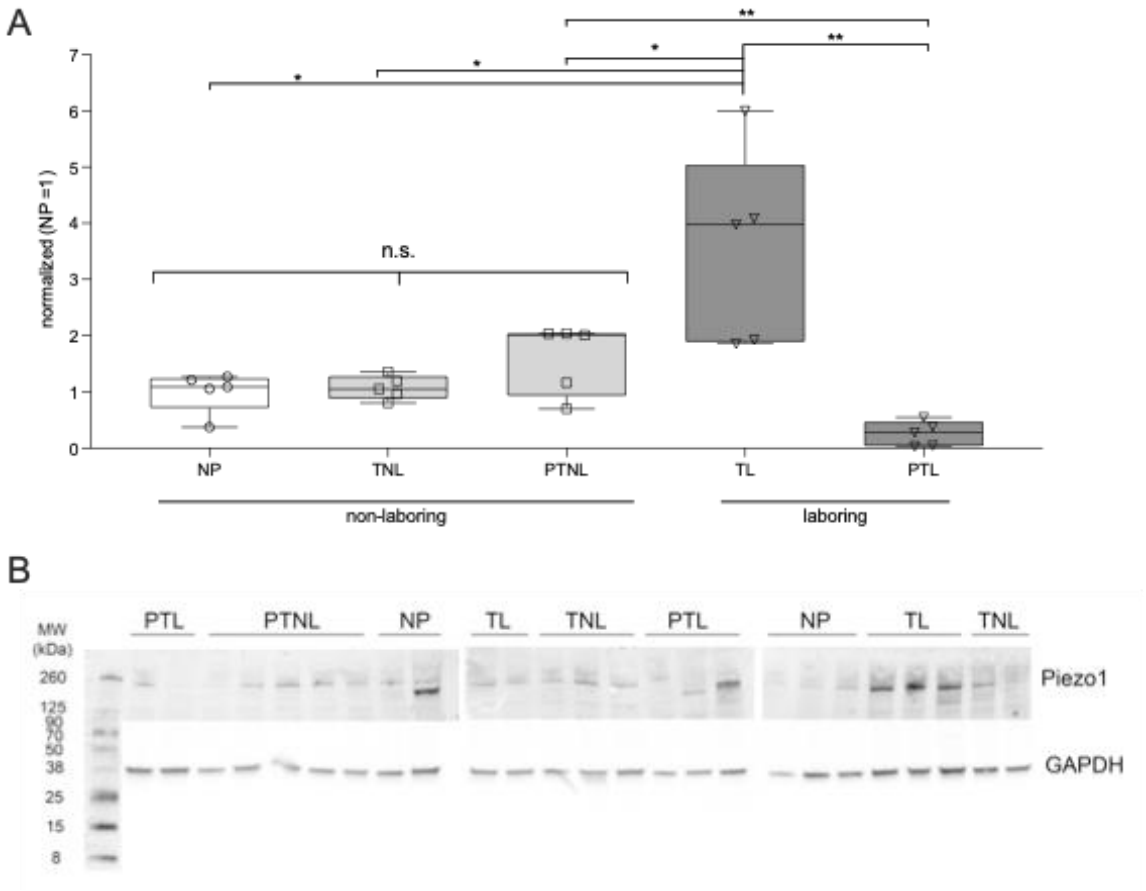

A
normalized (NP =1)
n.s.
NP
TNL
PTNL
TL
PTL
non-laboring
laboring
B
MW (kDa)
260
125
90
70
50
38
25
15
8
PTL
PTNL
NP
TL
TNL
PTL
NP
TL
TNL
Piezo1
GAPDH

Figure 1: Piezo1 protein expression in human myometrium – *A*, box and whisker plot (min–max) with overlay of individual values of western blot data using whole human myometrial tissue ($n = 5$ per state). Piezo1 expression does not significantly increase prior to labour (one-way ANOVA, $P = 0.103$); however, it is upregulated ~3.5-fold during TL ($P = 0.012$) *vs*. NP tissue and downregulated by ~14-fold in PTL relative to TL ($P = 0.0028$). Piezo1 expression is also significantly different in TL *vs*. PTNL ($P = 0.0427$), TL *vs*. TNL ($P = 0.0126$), PTNL *vs*. PTL ($P = 0.0020$) and TL *vs*. PTL ($P = 0.0028$). Each sample was normalized to GAPDH expression, and the average value of NP tissue was set to a nominal value of '1' for comparative purposes. NP, non-pregnant; PTL, preterm labouring; PTNL, preterm non-labouring; TL, term labouring; TNL, term non-labouring. *B*, western blots probed for Piezo1 (~260 kDa) and normalized to GAPDH.

Table 1. Pregnancy data

Sample type	Maternal age (years)	Gestational period (weeks)	Race	Complications
NP	41.8 ± 9.28	n/a	Caucasian (5)	Dysmenorrhoea; menorrhagia; abnormal uterine bleeding; omentum mass; endometriosis
TNL	29.0 ± 6.60	38.8 ± 1.10	Caucasian (5)	Cervical dilation; low fetal heart rate
TL	34.6 ± 6.19	38.6 ± 0.89	Caucasian (5)	Fetal macrosomia; failure to descend; fetal distress; uterine fibroids
PTNL	29.0 ± 6.78	32.8 ± 1.30	Latino (1), Caucasian (4)	Placenta previa; cervical intraepithelial neoplasia; preeclampsia; PPROM
PTL	29.6 ± 7.09	34.6 ± 1.52	Latino (1), Hispanic (1), Caucasian (3)	Cervical dilation, placenta accreta; placenta previa; breach

Table 1: Patient demographic - Maternal ages, gestational periods, race and pregnancy/birth complications presented for samples used in western blots. The difference in gestation between TL and PTL was 4 weeks. Data presented as means ± SD. PPROM, preterm premature rupture of membranes.

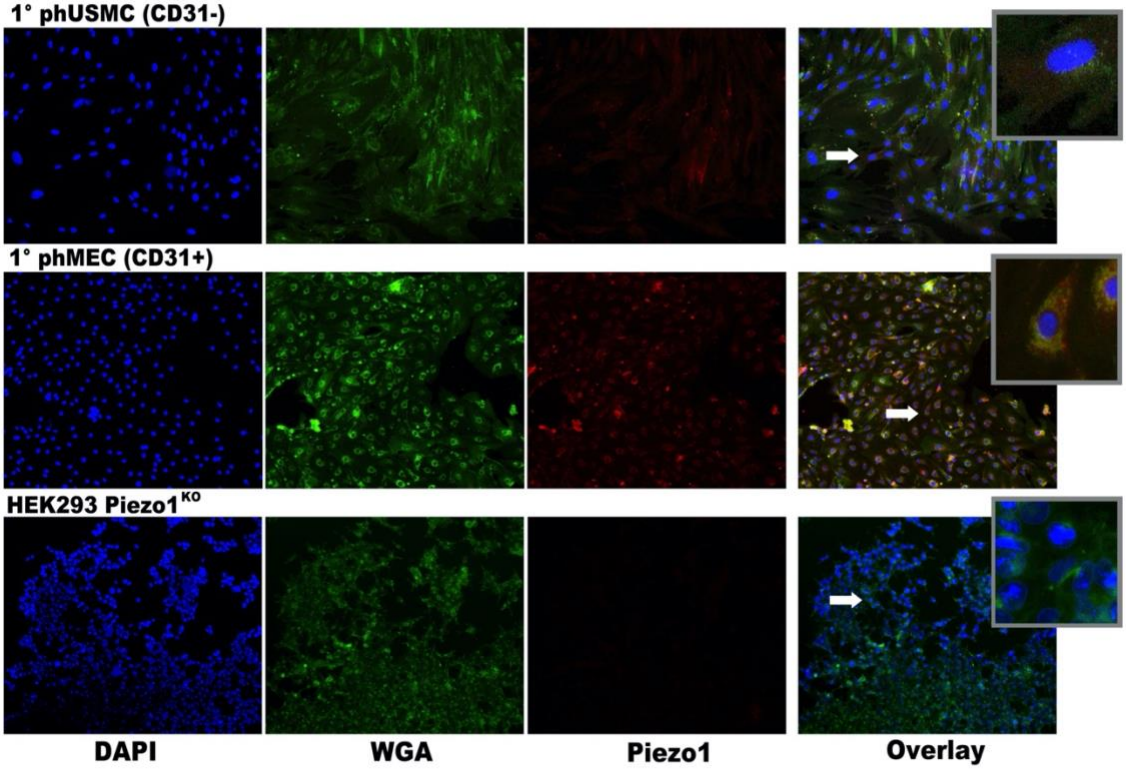
DAPI (*blue*), wheat germ agglutinin (WGA) to identify cellular boundaries (*green*), and Piezo1 (*red),* then imaged at 10x (Fig. 2A). Piezo1^{KO} cells were used as a negative control. *Western blot*: TNL human myometrial protein was collected from primary phMECs, phUSMCs (p2-4) and Piezo1^{KO} cells (n=4). Total protein was run on a Western blot (as described above) and labeled with Piezo1 antibody and normalized to GAPDH. phMECs exhibited a 2.89-fold increase (P=0.0008) in Piezo1 expression over phUSMCs, while Piezo1^{KO} cells expressed significantly less Piezo1 than either phUSMC (P=0.0185) or phMEC (P<0.0001) cell lines (Fig. 2B).

Piezo-mediated Ca^{2+} influx in CD31+ and CD31- cells

Piezo1 is selectively permeable to some monovalent and divalent cations (Coste et al. 2010b), primarily Ca^{2+} (Yuhao Zhang et al. 2021), with K^{+} permeability decreasing markedly in the presence of competing extracellular Ca^{2+} (Gnanasambandam et al. 2015). Because Piezo1 is expressed in both myometrial phMEC and phUSMC, we sought to quantify the relative Ca^{2+} permeability of Piezo1 in each cell type. To achieve this, we implemented an intracellular calcium flux assay following Piezo1 stimulation using the Piezo1 agonist Yoda1 (Cat. No. 5586 - Tocris, Minneapolis, MN). Calbryte™ 520 AM is a membrane permeable fluorescent Ca^{2+} indicator that becomes active when hydrolyzed by intracellular esterase. Myometrial-derived primary phUSMC/phMEC, and Piezo1^{KO} cells, were plated to ~80% confluency (Fig. 3D) and incubated with Calbryte™, followed by the addition of 2.5 mM Ca^{2+}, then exposed to either 0.3 or 3 µM Yoda1 ± 10 µM of

Fig. 2

A


1° phUSMC (CD31-)
1° phMEC (CD31+)
HEK293 Piezo1KO
DAPI
WGA
Piezo1
Overlay

B

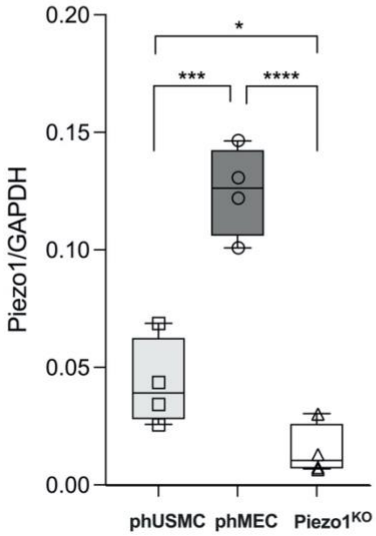

0.20
0.15
0.10
0.05
0.00
Piezo1/GAPDH
*
***
****
phUSMC
phMEC
Piezo1KO

Figure 2: Piezo1 expression in human myometrial phMEC, phUSMC and Piezo1^{KO} cells - *A*, immunofluorescence (IF) imaging of primary phUSMC and phMEC cells from TNL human myometrium and HEK293 Piezo1 KO cells, labelled with DAPI (nuclear), wheat germ agglutinin (WGA, membrane) and Piezo1-congugated antibodies. All images aquired at 10x magnification (scale bar shown) and areas of interest. *B*, box and whisker plot (min–max) of western blot data with overlay of individual values in myometrial phUSMC, phMEC, and Piezo1^{KO} cell lysates (n = 4) reveals a ~2.9-fold increase in Piezo1 expression in phMEC cells over phUSMC (P = 0.0008). Piezo1^{KO} cells expressed insignificant Piezo1 when compared to either phUSMC (P = 0.0185) or phMEC (P < 0.0001).

Fig. 3

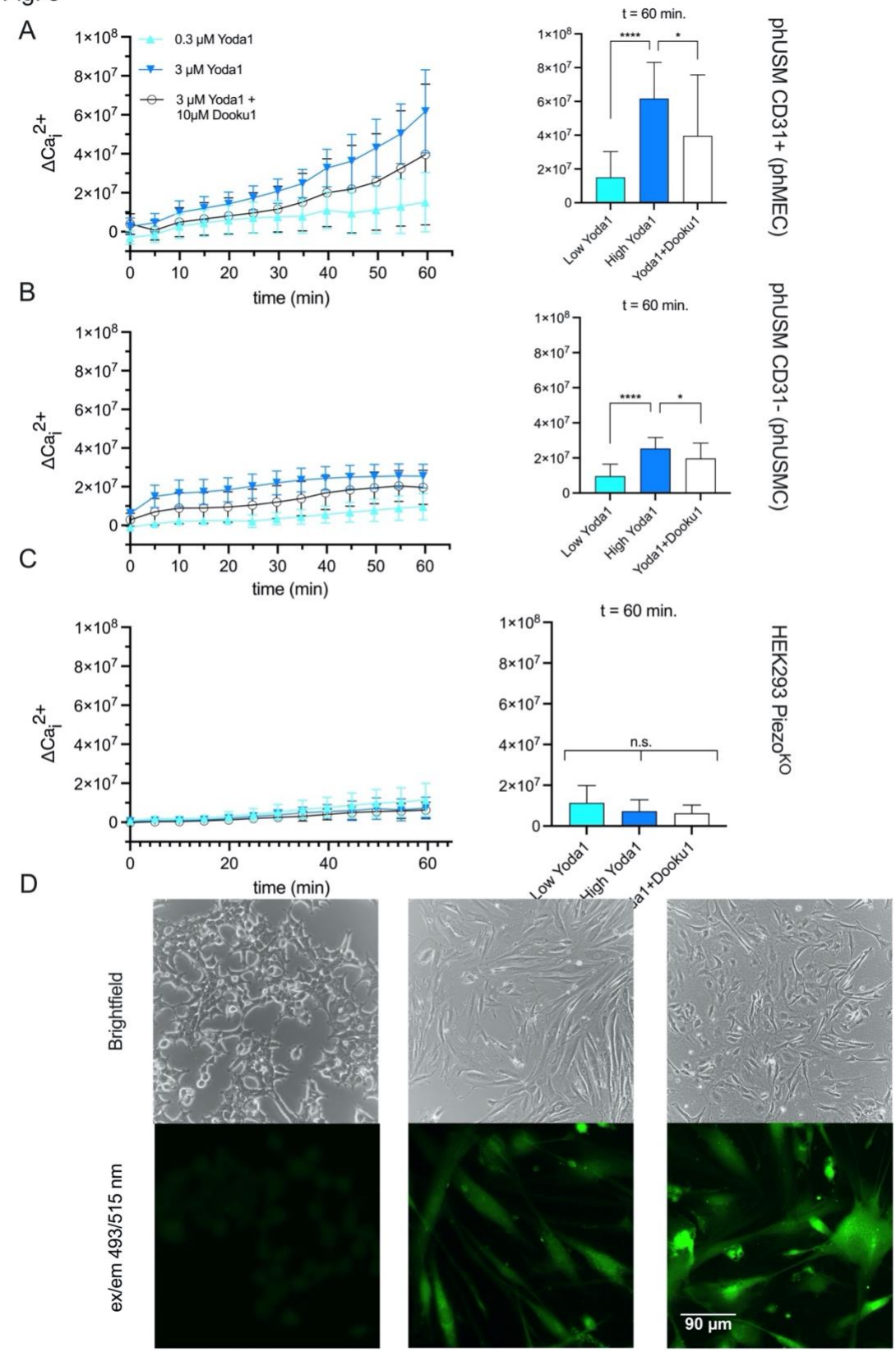

A
B
C
D
0.3 µM Yoda1
3 µM Yoda1
3 µM Yoda1 +
10µM Dooku1
ΔCa$_i^{2+}$
time (min)
t = 60 min.
****
*
n.s.
Low Yoda1
High Yoda1
Yoda1+Dooku1
phUSM CD31+ (phMEC)
phUSM CD31- (phUSMC)
HEK293 Piezo^{KO}
Brightfield
ex/em 493/515 nm
90 µm

Figure 3: Piezo1-meidiated Ca^{2+} influx in $CD31^{+}$ and $CD31^{-}$ human myometrial cells - An intracellular calcium flux assay determined Piezo1 activity in phMEC ($CD31^{+}$) and phUSMC ($CD31^{-}$) cells. phMEC, phUSMC and HEK293 $Piezo1^{KO}$ cells were pre-treated with the Ca^{2+} indicator Calbryte (ex/em 493/515 nm) followed by exposure to Yoda1 (0.3 or 3 μM) ± the Piezo1 antagonist Dooku1 (10 μM) and the change in fluorescence (ΔCa_i^{2+}) was measured. *A*, phMECs treated with 3 μM Yoda1 exhibited 4.09-fold increase in Ca^{2+} uptake (ΔCa_i^{2+}) over 0.3 μM treated cells ($P < 0.0001$) which decreased by 35.74% when co-treated with Dooku1 ($P = 0.0327$) at 60 min. *B*, phUSMC experienced a 2.64-fold increase in fluorescence when challenged with 3 μM Yoda1 ($P < 0.0001$), with a respective decrease of 22.49% when co-treated with Dooku1 ($P = 0.0326$). *C*, $Piezo1^{KO}$ fluorescence did not vary significantly at any dose of Yoda1 or Yoda1 + Dooku1 relative to baseline (Kruskal–Wallis one-way ANOVA, $P = 0.2622$). *D*, left, ×10 bright field images of phMEC (top), phUSMC (middle), and $Piezo1^{KO}$ (bottom). Right, Calbryte-induced fluorescence after 3 μM Yoda1 stimulation. ×20 fluorescence images of phMEC (top), phUSMC (middle), and $Piezo1^{KO}$ (bottom). Data presented as ±SD.

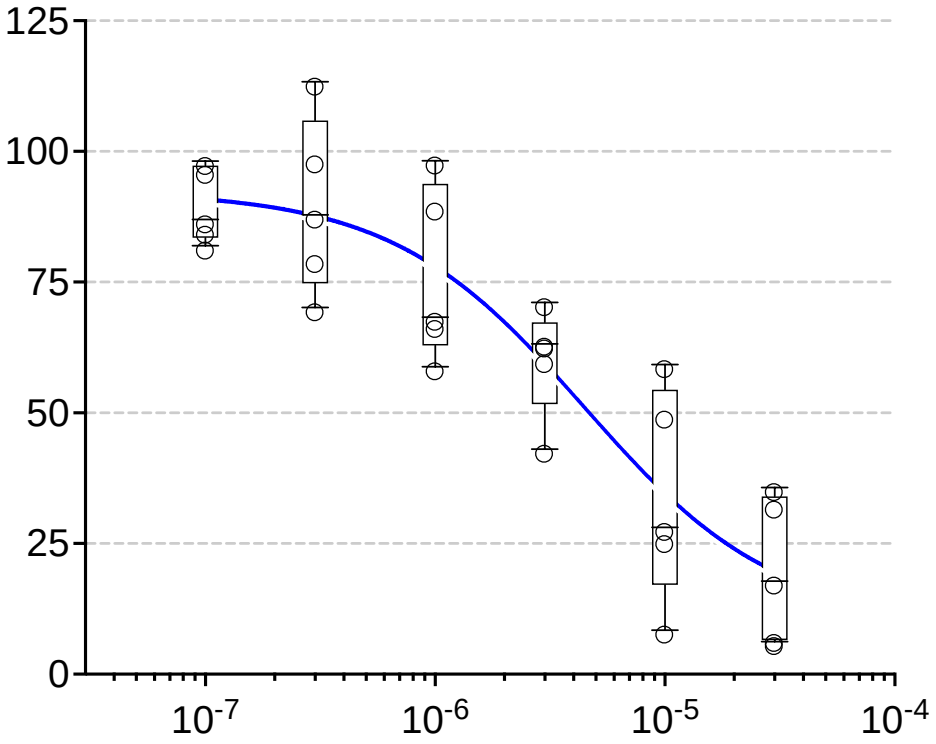
the Piezo1 antagonist, Dooku1. Fluorescence was recorded for 60 minute (ex/em 493/515 nm) at 5 minute intervals (n=18, Fig. 3A-3C). At the terminal time point (t=60 min), phMECs that had been treated with 3 μM Yoda1 (maximum dose) exhibited 4.09-fold increase in Ca^{2+} uptake (ΔCa_i^{2+}) over 0.3 μM treated cells (Fig. 3A, $p<0.0001$), which decreased by 35.74% when co-treated with Dooku1 ($P=0.0327$). phUSMC cells under the same conditions experienced a 2.64-fold increase in fluorescence ($p<0.0001$), with a respective decrease of 22.49% when co-treated with Dooku1 (Fig. 3B, $P=0.0326$). Conversely, in the $Piezo1^{KO}$ cell line, fluorescent signal did not vary significantly at any dose of Yoda1 or Yoda1 + Dooku1 relative to baseline (Fig. 3C, Kruskal-Wallis one-way ANOVA, $P=0.2622$).

EC_{50} of Yoda1 in human myometrium

The role of Ca^{2+} varies greatly throughout the body. In myometrial tissue Ca^{2+} entry into endothelial cells initiates a signaling cascade that activates endothelial nitric oxide synthase (eNOS), producing the quiescent-promoting molecule nitric oxide. In the myocyte its actions are more nuanced. While it is primarily known as a depolarizing molecule that triggers smooth muscle myosin phosphorylation (pMYL9) via calmodulin/MLCK activation, it also serves as a ligand to channels such as BK_{Ca}, which re-polarizes the cell through potassium efflux. To determine the net inotropic effect on whole myometrial tissue, human TNL myometrium (n=6) was exposed to an accumulative dose of Yoda1 in 15 minute intervals from 100 nM to 30 μM in half-log increments (Fig. 4). Each bath was normalized to itself upon return to normal, phasic contractions following oxytocin addition, then to a control bath at each time point (volumetric DMSO

Fig. 4

A


125
100
75
50
25
0
10-7
10-6
10-5
10-4

B

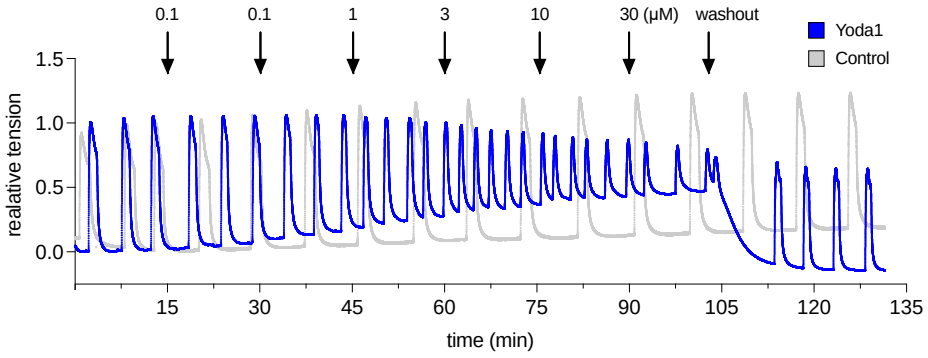

0.1
0.1
1
3
10
30 (μM)
washout
Yoda1
Control
1.5
1.0
0.5
0.0
realative tension
15
30
45
60
75
90
105
120
135
time (min)

Figure 4: EC_{50} of Piezo1 agonist (Yoda1) in human myometrium - *A*, TNL human myometrium (*n* = 6) was hung in an organ bath, oxytocin-challenged (8 nM), then dosed with the Piezo1 agonist Yoda1 at 15 min intervals (0.1, 0.3, 1, 3, 10, 30 μM). Tissue relaxed to Yoda1 exposure in a dose-dependent manner, with an EC_{50} of 3.02 μM. Relative to untreated control tissue (100%), the AUC for each concentration of Yoda1 was: 100 nM (88.03%, SD 13.01), 300 nM (86.72%, SD 18.65), 1 μM (70.91%, SD 15.52), 3 μM (52.02%, SD 15.50), 10 μM (35.08%, SD 5.62), 30 μM (29.74%, SD 16.59). Data presented as a box and whisker plot (5–95 percentile) with individual data point overlay. *B*, representative traces of Yoda1 and control tissue.

equivalent) to account for tissue rundown. The EC_{50} of Yoda1 in human myometrium, which we define as a reduction in the area under the curve (AUC) by 50% using the last three contractions per experimental period, was 3.02 µM with a corresponding Hill slope of -1.242 (Fig. 4). Relative to untreated control tissue (100%), the AUC for each concentration of Yoda1 was: 100 nM (88.03%, SD 13.01), 300 nM (86.72%, SD 18.65), 1 µM (70.91%, SD 15.52), 3 µM (52.02%, SD 15.50), 10 µM (35.08%, SD 5.62), 30 µM (29.74%, SD 16.59). Of note, this experiment was also run with the tissue stretched to a final tension of 1 gram (rather than 2 grams) to test for potential variability in Piezo1 activation under different amounts of stretch. No statistical difference in the dose-response curve was observed (*data not shown*).

The effect of PKA/AKT/eNOS/BK_{Ca} modulation on Yoda1-induced myometrial quiescence

Following the derivation of the EC_{50} for Yoda1 in human myometrium, we next opted to modulate endothelial Ca^{2+}-mediated pathways of nitric oxide generation, as well as the Ca^{2+}-activated BK_{Ca} channel, to determine their effects on Piezo1 stimulation by Yoda1. Both protein kinase A (PKA) and protein kinase B (AKT) are known to activate eNOS in MECs and can be stimulated through a rise in intracellular Ca^{2+}(Bir et al. 2012; X. P. Zhang and Hintze 2006). We elected to modulate PKA, AKT, eNOS, to determine whether these treatments would dampen the effects of Piezo-1 stimulation on the tissue, and to inhibit/activate BK_{Ca} during Piezo-1 stimulation to determine if BK_{Ca} contributes to Yoda1-induced quiescence (Fig. 5).

Fig. 5

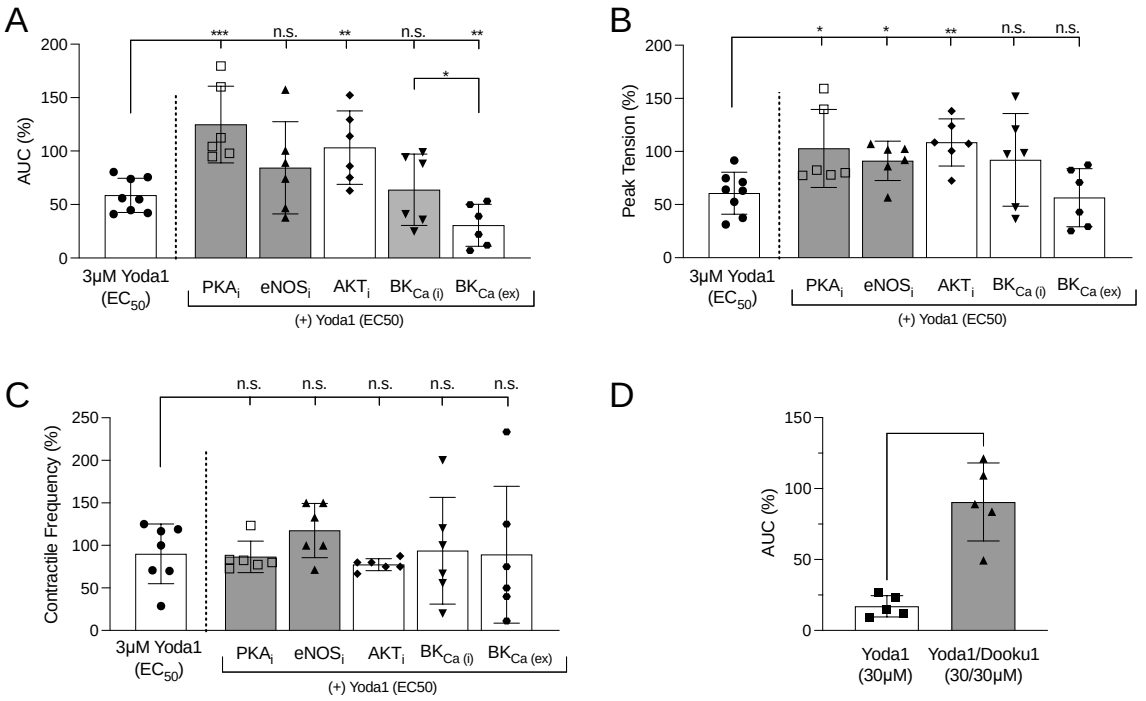

A
AUC (%)
200
150
100
50
0
***
n.s.
**
n.s.
**
*
3μM Yoda1 (EC50)
PKAi
eNOSi
AKTi
BKCa (i)
BKCa (ex)
(+) Yoda1 (EC50)
B
Peak Tension (%)
*
*
**
n.s.
n.s.
C
Contractile Frequency (%)
250
n.s.
D
AUC (%)
150
Yoda1 (30μM)
Yoda1/Dooku1 (30/30μM)

Figure 5: Inotropic effects of Piezo1 agonism on Ca^{2+}-mediated myometrial quiescent pathways - Using an organ bath (*n* = 6), area under the curve (AUC), peak tension and contractile frequency were determined after co-administration of an EC_{50} dose (3 μM) of Yoda1 + *myr*$PKI_{14–22}$ (PKA_i), FPA-124 (AKT_i), l-NNA ($eNOS_i$), Paxilline ($BK_{Ca(i)}$), or NS1619 ($BK_{Ca(ex)}$). *A*, AUC. In TNL myometrium, co-treatment with *myr*$PKI_{14–22}$ (10 μM) + Yoda1 (*P* = 0.0005) or FPA-124 (10 μM) + Yoda1 (*P* = 0.0067) significantly reduced the effects of Yoda1 returning AUC to baseline. Excitation of BK_{Ca} (NS1619, 30 μM) + Yoda1 resulted in an additive effect, decreasing AUC to 28.8% of baseline. *B*, peak tension. Co-treatments with *myr*$PKI_{14–22}$ + Yoda1 (*P* = 0.0026), FPA-124 + Yoda1 (*P* = 0.0021) and l-NNA + Yoda1 (*P* = 0.0402) resulted in a significant increase peak tension when compared to treatment with 3 μM Yoda1 alone, while neither NS1619 + Yoda1 (*P* = 0.4770) nor Paxilline + Yoda1 (*P* = 0.1887) significantly altered tension beyond that of 3 μM Yoda1 alone. *C*, contractile frequency. None of the treatment conditions, *myr*$PKI_{14–22}$ + Yoda1 (*P* = 0.8343), FPA-124 + Yoda1 (*P* = 0.4077), l-NNA + Yoda1 (*P* = 0.1718), NS1619 + Yoda1 (*P* = 0.8074), or Paxilline + Yoda1 (*P* = 0.7084), significantly changed contractile frequency when compared to a stand-alone EC_{50} dose of Yoda1. *D*, selectivity of Yoda1 was determined by comparing the AUC (*n* = 5) after a maximum dose of Yoda1 (30 μM) relative to co-treatment of Yoda 1 with Piezo1 antagonist, Dooku1 (30 μM/30 μM). Treatment with 30 μM Yoda1 decreased AUC to 17.08% of baseline, while co-treatment with Yoda1 + Dooku1 (30 μM/30 μM) recovered AUC to 90.55% of baseline (*P* = 0.0004).

As above, TNL human myometrium (n=6) was hung in an organ bath then pretreated for 15 minutes with the either the PKA inhibitor, 'PKI 14-22 amide, myristoylated' (*myr*PKI_{14-22} 10 µM) , the AKT inhibitor FPA-124 (10 µM) the eNOS inhibitor N_{ω}-Nitro-L-arginine, (L-NNA 100 µM), the BK_{Ca} inhibitor, Paxilline (10 µM, Cat. No. 2006, Tocris), or the BK_{Ca} activator, NS1619 (30 µM, Cat. No. 3804, Tocris), ± 3 µM Yoda1 (~EC_{50}) for 60 minutes. Area under the curve (AUC), peak tension, and contractile frequency were quantified.

AUC: Co-treatment of the tissue with *myr*PKI_{14-22} + Yoda1 (P=0.0005), FPA-124 + Yoda1 (P=0.0067), but not L-NNA + Yoda1 (P=0.3244) resulted in a significant increase AUC when compared to treatment with 3 µM Yoda1 alone. Stimulation of BK_{Ca} with NS1619 + Yoda1 exhibited an additive negative inotropic effect, further decreasing AUC to 28.83% of baseline (P=0.0077), while BK_{Ca} inhibition using Paxilline + Yoda1 (P=0.4466) did not significantly alter AUC beyond the effects of Yoda1 alone; however, NS1619/Yoda1 vs. Paxilline/Yoda1 were significantly different (P=0.0311) (Fig. 5A, right most two conditions), and the administration of either Paxilline or NS1619 as individual agents in the absence of Yoda1 produced significantly higher AUCs (105.1% and 82.86% of baseline, respectively) than when coadministered with Yoda1 (Paxilline vs. Paxilline + Yoda1, -41.34% ± 17.36, p=0.0206; NS1619 vs. NS1619 + Yoda1, -52.32% ± 21.27, p=0.0181 - data not shown). AUC was also determined using a maximum dose of Yoda1 (30 µM) and analyzed against co-treatment with the Piezo1 antagonist, Dooku1 (30 µM/30 µM), to ensure the selectivity of Yoda1. Treatment for 60 minutes with 30 µM Yoda1 decreased AUC to 17.08% of baseline, while co-

treatment with Yoda1 + Dooku1 (30 µM/30 µM) recovered AUC to 90.55% of baseline (Fig. 5D, P=0.0004).

Peak Tension: In contrast to AUC, co-treatment of the tissue with *myr*PKI_{14-22} + Yoda1 (P=0.0026), FPA-124 + Yoda1 (P=0.0021), and L-NNA + Yoda1 (P=0.0402) resulted in a significant increase peak tension, while neither BK_{Ca} stimulation with NS1619 + Yoda1 (P=0.4770), nor inhibition with Paxilline + Yoda1 (P=0.1887), had a significant effect on tension when compared to treatment with 3 µM Yoda1 alone (Fig. 5B).

Contractile frequency: Co-treatment of the tissue with *myr*PKI_{14-22} + Yoda1 (P=0.8343), FPA-124 + Yoda1 (P=0.4077), L-NNA + Yoda1 (P=0.1718), NS1619 + Yoda1 (P=0.8074), or Paxilline + Yoda1 (P=0.7084), did not alter contractile frequency (Fig. 5C).

Discussion

The two primary findings of this research are the novel identification of the mechanosensitive cation channel, Piezo1, in human myometrium, and the discovery that Piezo1 stimulation imparts negative inotropic effects on the tissue, intimating its potential as a tocolytic (Fig. 6).

Spontaneous PTL remains largely an enigma. Advances in perinatal therapeutics bely our failure to better understand the most relevant contributing factors that initiate idiopathic labor. To advance our understanding of myometrial quiescence we posit that it is necessary to view the myometrium simplistically as a two-compartment system. We do not disavow the importance of resident immune cells, nor their role in inflammatory regulation of pregnancy, rather we are exploring relaxation mechanisms that can be demonstrated to prevent PTL in the face of the inflammatory environment.

In many ways it is reasonable to classify pregnancy as a transient pathological state. Following a marked and prolonged increase in proinflammatory mediators during pregnancy (Cappelletti et al. 2016; Mor et al. 2011), intense stretching of the uterine cavity often initiates labor (Waldorf et al. 2015), and it has been theorized that the resulting labor is amplified through the phasic increase in uterine load during subsequent contractions forming a positive feedback loop (Roger C. Young 2016). Practically speaking, this is realized by the uptick of PTB rates observed with multi-fetal pregnancies (twins 60.87%, triplets 98.5%) (Martin et al. 2021). Given the unique hydrostatic loads endured by the myocytes and

Fig. 6

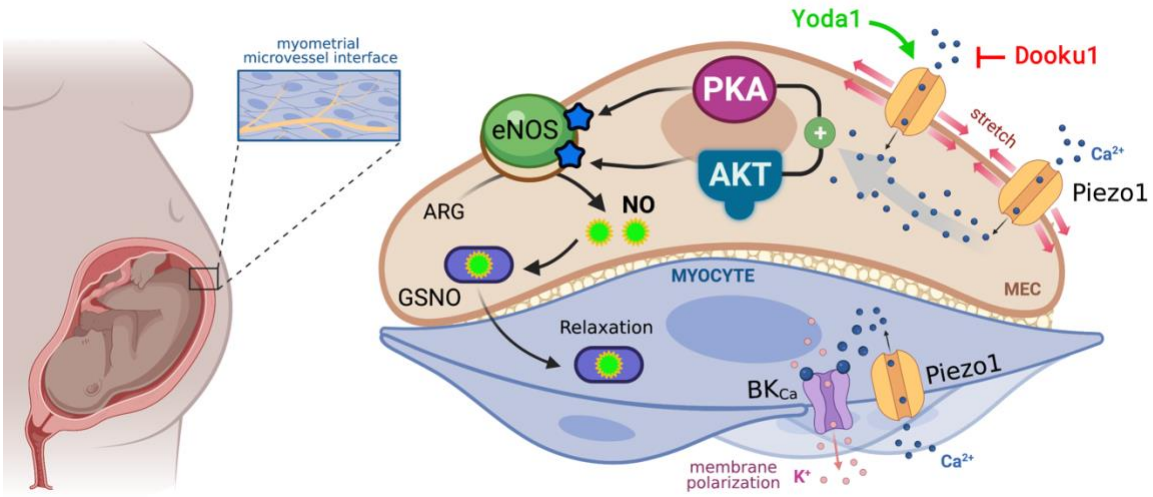

myometrial
microvessel interface
Yoda1
Dooku1
PKA
eNOS
AKT
ARG
NO
GSNO
stretch
Ca2+
Piezo1
MYOCYTE
MEC
Relaxation
BKCa
Piezo1
membrane
polarization
K+
Ca2+

Figure 6: Proposed pathway for Piezo1-mediated quiescence in human myometrium - We posit that the stretch experienced by the uterus as the fetus develops activates Piezo1 channels on endothelium and smooth muscle cells of the myometrium. This stretch drives Ca^{2+} influx into the endothelium where PKA and AKT activate eNOS, generating the quiescence-promoting molecule, nitric oxide, which migrates to the myocyte in the form of *S*-nitrosoglutathione (GSNO). Concurrently, in the smooth muscle, Piezo1 activation may contribute to BK_{Ca} activation, which polarizes the membrane through K^+ efflux. Graphic created with BioRender.com.

microvascular endothelium of the myometrium during pregnancy, stretch activated channels (SACs) present as a logical investigative target.

Many important contractile proteins are regulated and/or activated by stretch. Cx43, a critical uterine gap junction that imparts cable properties to the myometrium during labor (Pierce et al. 2002; Scott D. Barnett, Asif, Anderson, et al. 2020), is upregulated by mechanical stress (Tellios et al. 2019; Y. Shi 2021). It has even been suggested that stretch activation of gap junctions in the cervix may initiate crosstalk between the cervix and myometrium to initiate labor (Vink 2020). TREK-1, an outward rectifying potassium channel is activated by, and upregulated by stretch (Buxton, Singer, and Tichenor 2010; Heyman et al. 2013), a finding amplified in twin pregnancies (Yin et al. 2018). When considering the role stretch and PTL, it is important to note that stretch alone is most likely not the primary driver of early labor, especially in singleton pregnancies. During ‘extremely preterm’ (less than 28 weeks) and ‘very preterm’ labor (28 to 32 weeks), the fetus and amnion have not developed enough to impart the excessive uterine strain needed to initiate labor, as evidenced by the finding that the ‘maximum uterine wall tension’ in PTL pregnancies (singleton) from weeks 20-30 are equal to or less than in pregnancies that carry to term (Sokolowski et al. 2010). Our finding that Piezo1 is differentially regulated between TL and TNL, and between PTL and PTNL (Fig. 1), indicates the Piezo1 expression is more likely linked to labor state, and not specifically uterine distension. If we consider that during active labor the myometrium must modulate rapidly between contraction and quiescence, it is not surprising that Piezo1 is upregulated to facilitate the phasic nature of the laboring

process, and the fact that Piezo1 is significantly downregulated in PTL vs TL suggest that Piezo1 is dysregulated in PTL myometrium. Further, because Piezo1 can be stimulated beyond what stretch alone can achieve using Yoda1 (Fig. 4), it is possible that chemical stimulation of the downregulated channel (PTL myometrium) may compensate for the dearth of Piezo1.

Our finding that Piezo1 is expressed in both the myocyte and microvascular endothelium of the myometrium (Fig. 2) necessitated a more thorough investigation into the role of Piezo1 in each cell type. There is a paucity of data in smooth muscle to determine the role of Piezo1 channel function (Retailleau et al. 2015), and no studies are available (National Library of Medicine, n.d.) examining Piezo1 channels in uterine smooth muscle, or their effect on tension.

Endothelial Piezo1 channels have been found to either relax (Evans et al. 2018), or contract (Rode et al. 2017), underlying smooth muscle depending on their tissue location and the presence of pathology. Ca^{2+} entry following activation of Piezo1 channels in endothelium leads to increased nitric oxide generation *via* eNOS activation through cAMP-mediated activation of PKA (Bir et al. 2012) or by phosphoinositide3-kinase (AKT) (X. P. Zhang and Hintze 2006). Piezo1 channels have not yet been studied in the endothelial cells of the myometrium. Their presence, as we demonstrate here, provides an explanation for the physiological origin of nitric oxide as a constitutive quiescence signal during gestation. When investigating expression of Piezo1 and the subsequent Ca^{2+} entry into phMECs via Piezo1 stimulation, we found that phMECs express Piezo1 at a higher level than phUSMCs (Fig. 2B), and that the expression of Piezo1 correlates to Ca^{2+}

permeability (Fig. 3A/3B). This implies that Piezo1's relative abundance on phMECs contributes to nitric oxide generation. Our further analysis of Piezo1 activation with Yoda1 using whole tissue in the organ bath supports this hypothesis, as the inhibition of PKA, AKT or eNOS each diminished the effects of Piezo1 stimulation by Yoda1 (Fig. 5).

Although Piezo1 is an inwardly rectifying cation channel, it cannot be assumed to contribute to contraction in myometrial muscle. Ca^{2+} influx in myometrium, mediated by voltage-dependent L-type Ca^{2+} channels (Bean 1989), which is blocked by nifedipine and amlodipine, is expected to determine the final contractile state of myometrium, and ultimately parturition (S Wray et al. 2003). However, calcium-channel blockers do not provide adequate tocolysis (van Vliet et al. 2016; Songthamwat, Na Nan, and Songthamwat 2018; Nijman et al. 2016) at safe doses. Smooth muscle Ca^{2+} signals can differ in spatial and temporal distribution (Amberg and Navedo 2013; Brozovich et al. 2016) and include highly localized Ca^{2+} release events (*e.g.,* sparklets and sparks) (Brozovich et al. 2016) that regulate signaling locally (Mercado et al. 2014); are not necessarily elicited by global increases in $[Ca^{2+}]i$; are compartmented (Buxton and Brunton 1983); and do not result in contraction *per se*, thus, we do not attribute phUSMC Piezo1 activation to providing a Ca^{2+} source for contraction. This is evidenced most notably by our finding that Piezo1 stimulation by Yoda1 induces relaxation of the myometrium in a dose-dependent manner (Fig. 4), and through our finding that BK_{Ca} stimulation amplifies the Yoda-1 response (Fig. 5A). Because the myometrium is ~90% muscle by volume, if Piezo1 stimulation primarily aided in

depolarization of the myocyte, we would not expect to see such robust negative inotropic effects on the tissue (Fig. 4, Fig.6), a finding amplified by our observation that Yoda1 was still effective at reducing AUC when eNOS was inhibited (Fig. 5A). While a shortcoming of this study was the limited availability of PTL myometrium for additional organ bath testing, the TNL myometrium used in lieu of PTL exhibits a comparable expression profile of Piezo1 (Fig. 1), inferring its potential as a suitable analog.

If Ca^{2+} permeation via Piezo1 in uterine myocytes does not facilitate contraction, how might it mediate quiescent signaling? It has previously been determined that stretch activation of Piezo1 results in stimulation of large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels (Hoyer et al. 1994; Jakob et al. 2021). BK_{Ca} mRNA (J. Shi et al. 2015) and protein (Wakle-Prabagaran et al. 2016) have been identified in uterine smooth muscle, and BK_{Ca} is regulated by pregnancy state (Gao et al. 2009). In the myometrium, BK_{Ca} stimulation via the mechanosensitive Ca^{2+} channel, TRPV4 (Liedtke 2006)**,** has already been shown to relax the tissue (Villegas et al. 2021). Our finding that peak tension is significantly reduced following co-administration of L-NNA (eNOS inhibition) + Yoda1 (Piezo1 stimulation) indicates that myocyte-bound Piezo1 likely contributes to BK_{Ca} stimulation (Fig. 5B). It is curious that inhibition of BK_{Ca} does not significantly alter the contractile dynamics of Yoda1 EC_{50}-dosed myometrium (Fig. 5 A-C); however, BK_{Ca} stimulation by NS1619 does further reduce AUC beyond the Yoda1 EC_{50}-dosed myometrium to approximately 25% of baseline. The failure of BK_{Ca} inhibition to alter the response to Yoda1 is not entirely unexpected as

Aaronson et al.,(Aaronson et al. 2006) examining BK_{Ca} inhibition in rat myometrium, noted the failure of BK_{Ca} channel inhibitors to significantly affect contractility in strips from either nonpregnant or pregnant animals. Nonetheless, activation of BK_{Ca} in the presence of Yoda1 suppresses contractions approximately 50% more that BK_{Ca} activation in the absence of Yoda1 (-52.32% ± 21.27 of control AUC). Because inhibition of BK_{Ca} in the absence of Yoda1 does not alter the response to oxytocin, it may be that more than one outwardly rectifying K+ channel contributes to contraction/relaxation dynamics and thus limits what can be resolved from BK_{Ca} modulation alone. Indeed, such compensation by channels such as TREK-1 are likely(B M Sanborn 2000; Heyman et al. 2013).

Taken together, our data imply an interplay between endothelium and muscle to regulate tone, and that disease can influence the expression of, and ability of Piezo1 channels to mediate contraction. We posit that there is a complex relationship between overlapping and compensatory mechanisms that facilitate the phasic contractile pathways studied here, to include pathways beyond the scope of this investigation. Such a notion is consistent with our hypothesis that myometrial Piezo1 normally provides a homeostatic function that modulates stretch-activated membrane hyperpolarization by cation influx without inducing contraction.

Preterm labor is a confounding obstetric dilemma. The exceptional physiology of the myometrium necessitates its complete quiescence for the 40 weeks of gestation, all while adapting to the increase in uterine strain as the pregnancy progresses. We have determined that the stretch-activated channel,

Piezo1, is expressed in the myocytes and microvascular endothelium of the myometrium, that it is dysregulated during PTL, and that its activation via Yoda1 results in myometrial quiescence through endothelial PKA/AKT activation of eNOS. Similarly, the β3AR is expressed in both myocytes and microvascular endothelium. Furthermore, the β3AR increases NO production through activation of eNOS and stimulates the BK_{Ca} as well (Asif, Barnett, and Buxton 2022). As such, although Piezo1 is stretch activated, it has similar downstream mechanisms of relaxation like the β3AR. Thus, Piezo1 emerges as a novel target for future tocolytic development.

Chapter 5

Conclusion and Future Directions

Conclusion

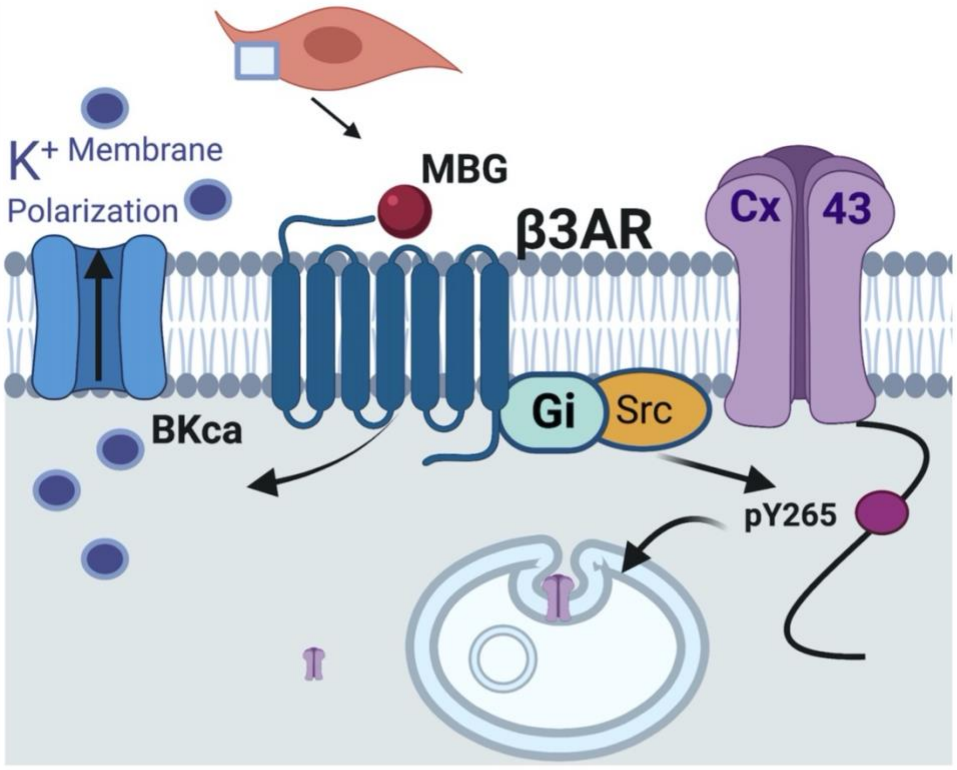
Gestation and labor during pregnancy are mediated by multiple factors, making it difficult to establish a proper treatment strategy for preterm labor (PTL) (Behrman and Butler 2007b). The search for an efficacious tocolytic strategy has been going on for decades, and current strategies may only delay labor for up to 48 hours (Haas et al. 2012). The tocolytic methods currently employed cannot be used for long periods of time due to adverse effects they may have on the fetus and mother (Katz and Farmer 1999). In order to develop efficacious strategies to combat PTL, we need a better understanding of the pathophysiological processes occurring within the myometrium before and during the time of labor and target mechanisms decrease the adverse effects on the fetus and mother.

The myometrium is unique relative to other smooth muscle systems. The myometrium relaxes to nitric oxide (NO) in a cyclic GMP independent manner, and it has been shown that the cyclic AMP and protein kinase A pathway have a limited role in mediating relaxation in the myometrium (Buxton et al. 2010; Lai et al. 2016). Thus, we investigated the β3 adrenergic receptor (β3AR) for its potentail to increase endogenous NO and use PKA independent pathways, and provided evidence for its potential as a tocolytic strategy.

An important fact that drives most of the research in this dissertation is that the β3AR is highly upregulated in disparate states of pregnancy (Chapter 2), and that it does not downregulate from over activation like β2 adrenergic receptors do (Schena and Caplan 2019). In these studies, we used the β3AR agonist, mirabegron (MBG), since it is a pregnancy category C drug that can be used during

Fig. 1

a.


K+ Membrane
Polarization
MBG
β3AR
Cx
43
BKca
Gi
Src
pY265

b.

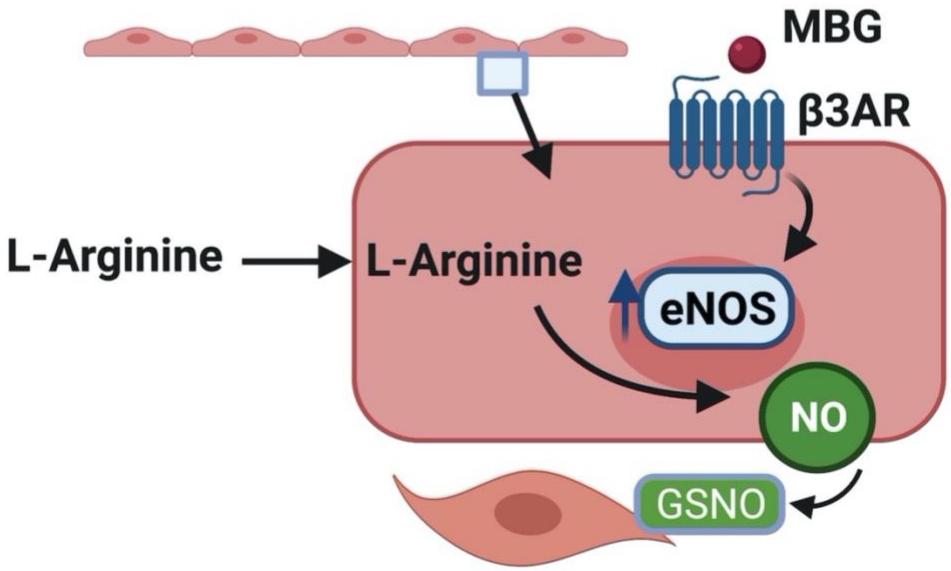

MBG
β3AR
L-Arginine
L-Arginine
eNOS
NO
GSNO

Figure 1: β3AR signaling in the human myometrium – a.) MBG activates β3AR to promote the opening of the BK_{Ca} for K^+ ions to move and polarize the cell. β3AR activation leads to Src phosphorylation of the Y265 site on Cx43, which promotes down regulation of the contractile associated protein. b.) β3AR activation from MBG increases NO production in myometrial endothelial cells for relaxation.

pregnancy and is already FDA approved for the relaxation of the bladder smooth muscle. Since it is FDA approved, regulatory approval for its use in clinical trials for preterm labor in the future may be accelerated.

We showed that MBG is capable of mediating relaxation in contracting uterine strips. In smooth muscle, we showed that MBG stimulates BK_{Ca} activation and down regulation of Cx43 (Chapter 2, Chapter 3). The BK_{Ca} helps polarize the membrane potential, while the down regulation of Cx43 decreases the intercellular communication for Ca^{2+} exchange (Fig. 1a). Both these factors are crucial for propagating contractions. We showed that inhibition of the Cx43 gap junction can be a potential target for mediating quiescence (Chapter 3). Since Cx43 plays a large role in cardiac function where β1 and β2 adrenergic receptors are the dominant subtype, we can use β3AR to target Cx43 in the myometrium during pregnancy to mitigate the risks for adverse effects.

In myometrial endothelial cells, we showed β3AR stimulation increases NO production to promote relaxation (Fig. 1b). We showed that NO can cause nitrosation of Cx43, which helps it maintain a hemichannel state and prevent gap junction formation (Chapter 3). Although NO has been shown to have a blunting effect in PTL (Scott D. Barnett et al. 2018), β3AR can utilize a combination of NO and the previous signaling pathways mentioned before to have a greater effect than NO produced alone. The multiple signaling pathways associated with β3AR make it a potential target for the development of tocolytic strategies.

Future Directions

Fig. 2

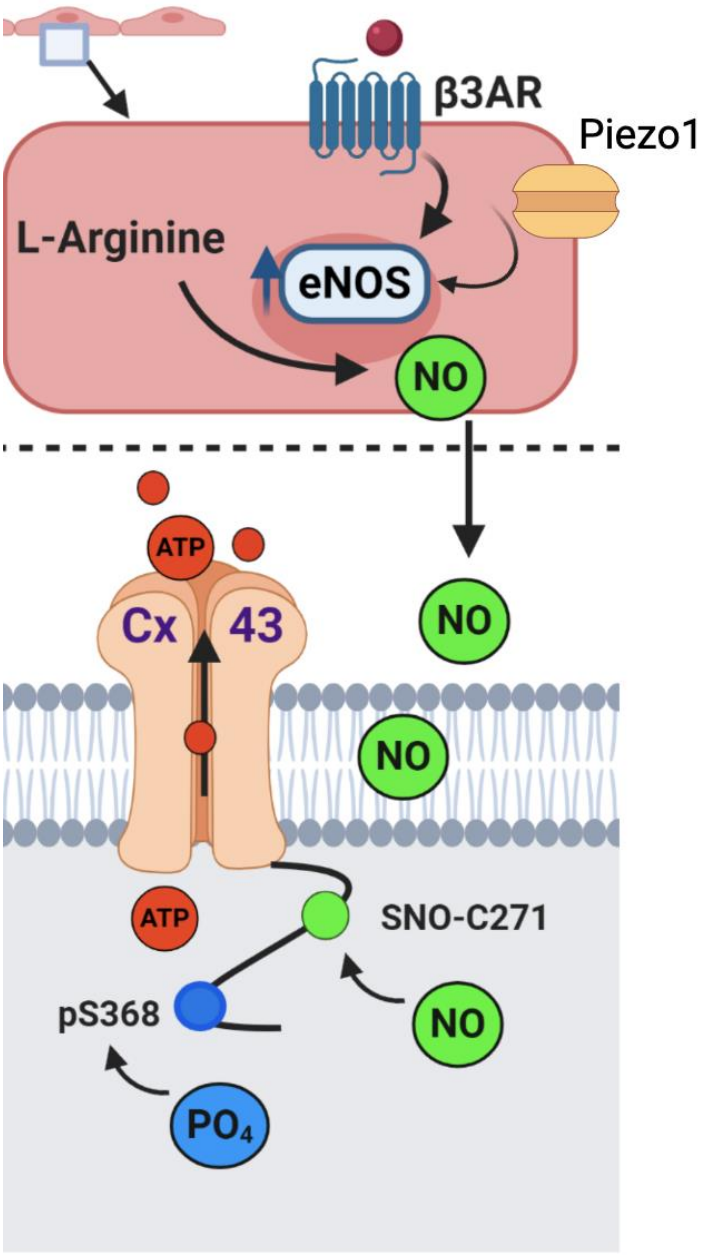


Figure 2: S-nitrosation of Cx43 through endogenous NO production – Coactivation of β3AR and Piezo1 may have an additive effect on NO production. Further research can deterine if the increase in endogenous NO can S-nitrosate Cx43. If nitrosated, it promtoes the phosphorylation of S368, which favors a hemichannel state for the Cx43.

Combination tocolytic therapy may be more effective in delaying preterm labor by inhibiting multiple contractile pathways for an additive effect (Kashanian et al. 2020). A benefit of combination tocolytic therapy is that if drugs have an additive effect, they can be effective at lower concentrations to achieve the same result while decreasing the likelihood of adverse effects from each. Along with β3AR, we identified the mechanosensitive Piezo1 channel to be present in both the myometrial smooth muscle and endothelium. Similar to the β3AR, Piezo1 activation increases NO production and stimulates the BK_{Ca} (Chapter 4). Since both β3AR and Piezo1 have similar downstream effects, they may have additive effects to promote relaxation. As mentioned earlier, we showed that NO can nitrosate Cx43 to promote a hemichannel state, and both Piezo1 and β3AR increase endogenous NO (Chapter 3, 4). Further studies can determine whether increasing endogenous NO from activation of these two receptors can directly promote the S-nitrosation of Cx43 (Fig. 2).

Previous studies from our lab showed that S-nitrosoglutathione reductase (GSNOR) is increased in laboring states of pregnancy, therefore potentially downregulating the NO mediated S-nitrosation of Cx43 (Chapter 3, Barnett et al. 2018). Since β3AR mediates relaxation using NO, a combination tocolytic therapy using both MBG and a GSNOR inhibitor (N6022) can upregulate nitrosation events, ulitmately increasing the relaxative effects of NO.

In Chapter 3, we showed that coadministration of nebivolol (partial β3AR agonist) and 18β-glycyrrhetinic acid (18β-GA, gap junction inhibitor) have additive

effects on contracting uterine strips. Further studies could determine if using a highly selective β3AR agonist, MBG, in combination with 18β-GA in combination tocolytic therapy could potentially have a higher efficacy.

In conclusion, the mechanistic pathways leading to the onset of PTL are not completely understoof and current tocolytic strategies are ineffective. Here we provided evidence for some mechanisms associated with β3AR mediated relaxation in the human myometrium, which include increasing NO production, activating BK_{Ca}, and down regulating Cx43. We showed that down regulation of Cx43 gap junctions may occur through either phosphorylation events or S-nitrosation in the human myometrium. Our research identifies Piezo1 as a potential target for tocolytic strategies utilizing similar mechanisms as β3AR, and suggests that Piezo1/ β3AR combination therapies may provide additivie effects. Overall, this research supports the use of β3AR agonist, MBG, as a potential candidate for developing novel tocolytic strategies, and provides insight into the pathophysiology of PTL.

Appendix A

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