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

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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.

Keywords

Myometrium; Preterm Labor; Pregnancy; Mechanosensitive channels; Stretch-activated channels; Piezo1

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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 & Ahmmad, 2021), and their infants twice as likely to die as a result (Burris & 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 & 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 & 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 (Sanders & Smith, 1986; Sanders et al., 2016), human myometrial muscle fibers are interwoven, do not form distinct layers (Blanks et al., 2007); are not innervated by motor nerves (Tingåker & Irestedt, 2010); and a pacemaker cell type has not been convincingly described (Young, 2018; Wray & Prendergast, 2019). These peculiarities of myometrium, combined with the finding that uterine myocytes preferentially relax to nitric oxide via protein S-nitrosation (Barnett et al., 2018), and not cyclic nucleotide generation (Bradley et al., 1998; Lai et al., 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*) (Buxton et al., 2011; Heyman et al., 2013), 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 (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 et al., 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 et al., 2012). It is selectively agonized/antagonized by the small molecules Yoda1 and Dooku1, respectively (Evans et al., 2018; Botello-Smith et al., 2019; 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 & 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 et al., 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 (Zhang & 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 (Barnett 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 et al. 2022). 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 & 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 (Wictome et al., 1992; Xuan 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(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 et al., 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$_{14\text{-}22}$ 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 1) 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, 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., 2010*b*), primarily Ca^{2+} (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 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 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+}(Zhang & Hintze, 2006; Bir et al., 2012). 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).

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 (Mor et al., 2011; Cappelletti et al., 2016), 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 (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 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; Barnett et al., 2020), is upregulated by mechanical stress (Tellios et al., 2019; 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 et al., 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) (Zhang & 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 (Wray et al., 2003). However, calcium-channel blockers do not provide adequate tocolysis (Nijman et al., 2016; van Vliet et al., 2016; Songthamwat et al., 2018) at safe doses. Smooth muscle Ca^{2+} signals can differ in spatial and temporal distribution (Amberg & 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 & 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 (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(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.

Conclusion:

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. As such, Piezo1 emerges as a novel target for future tocolytic development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Biography

Scott Barnett received his Ph.D. in Cellular and Molecular Pharmacology & Physiology from the University of Nevada, Reno, where he studied the pathophysiology of preterm labor. Upon completion of his degree, Dr. Barnett joined the Medical College of Wisconsin as a PhRMA Foundation Postdoctoral Fellow where he investigated the treatment of renal diseases using dual-ligand pharmacology. Dr. Barnett currently resides in the laboratory of Dr. Iain. L.O. Buxton at the University of Nevada, Reno School of Medicine (Pharmacology), where he identifies and explores dysregulated myometrial pathways to aid in novel tocolytic development.

Data availability statement:

All data in a non-identifying format are securely stored at the University of Nevada, Reno. The data are available upon request.

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Key Points:

- Spontaneous preterm labor is a serious obstetric dilemma without a known cause or effective treatments.
- Piezo1 is a stretch-activated channel important to muscle contractile dynamics.
- Piezo1 is present in the myometrium and is dysregulated in women who experience preterm labor.
- Activation of Piezo1 by the agonist, Yoda1, relaxes the myometrium in a dose-dependent fashion, indicating that Piezo1 modulation may have therapeutic benefits to treat preterm labor.

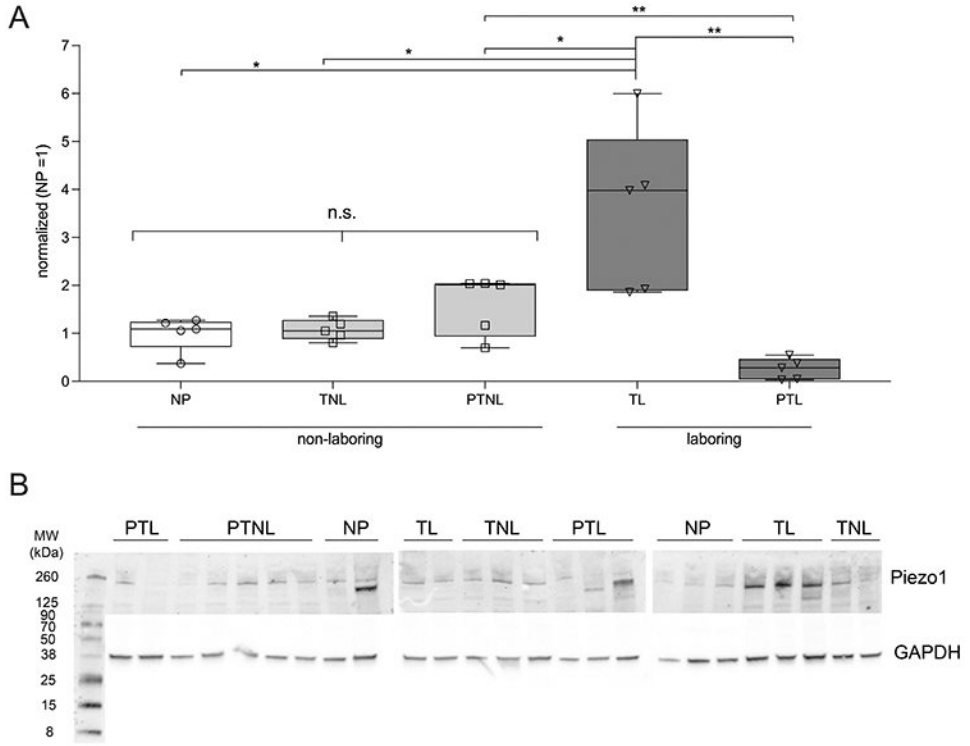

figure 1: Piezo1 protein expression in human myometrium

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 labor (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; TNL - term non-laboring; TL - term laboring; PTNL - preterm non-laboring; PTL - preterm laboring. B) Western blots probed for Piezo1 (~260 kDa) and normalized to GAPDH.

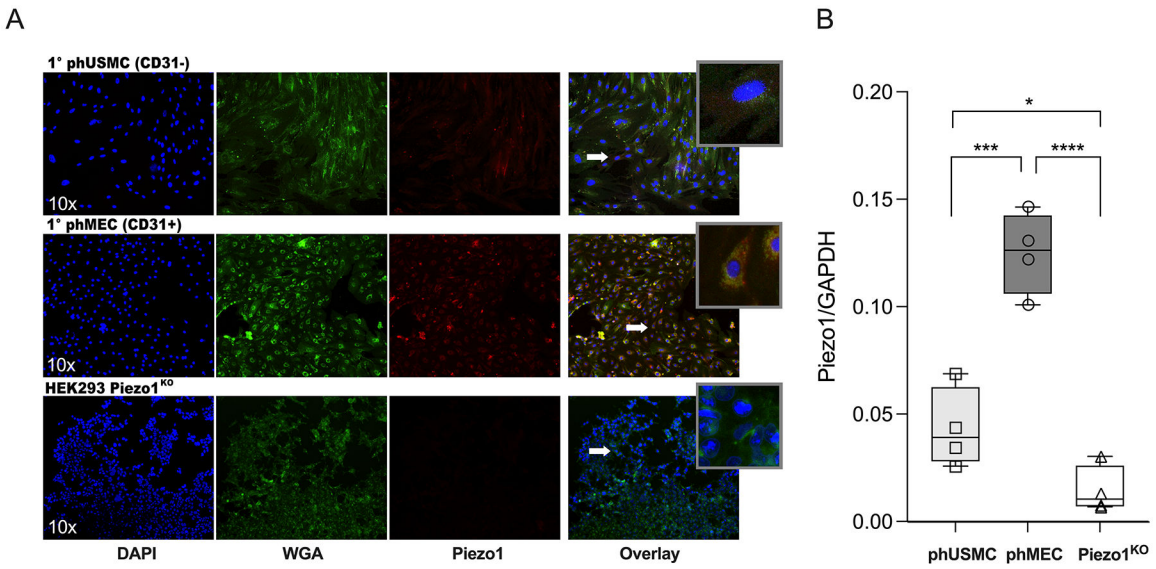

figure 2: Piezo1 expression in human myometrial phMEC, phUSMC and Piezo1^{KO} cells

A) Immunofluorescent (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. Magnified areas of interest (white arrows) presented in the overlay 'inset'. 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).

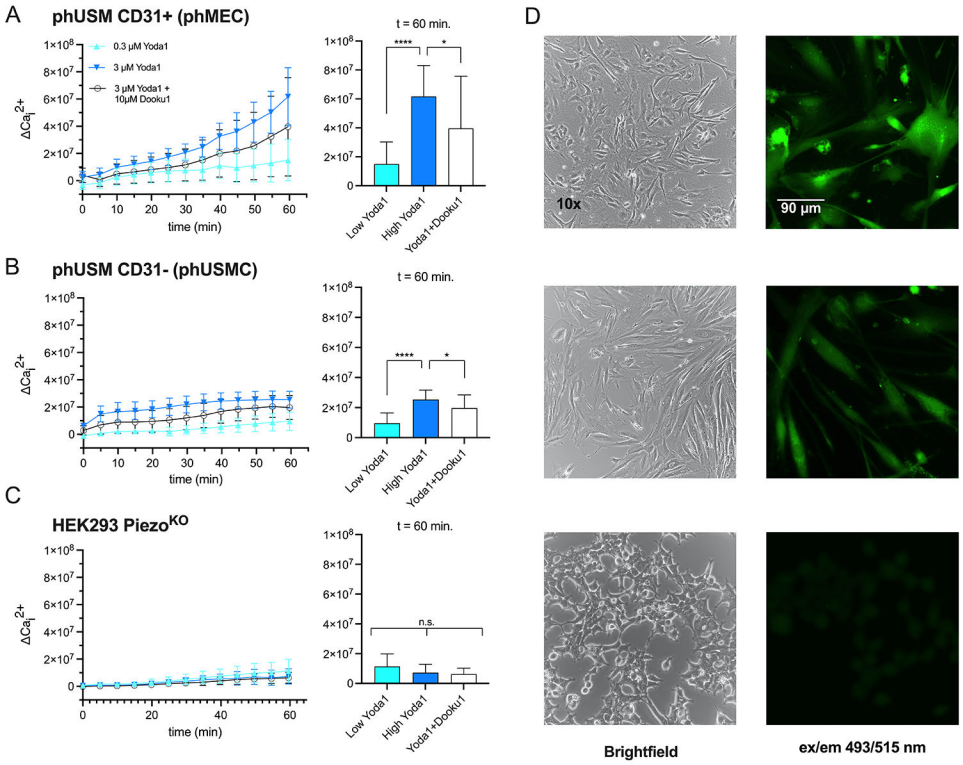

Figure 3: Piezo1-meidiated Ca^{2+} influx in CD31+ and CD31− human myometrial cells

An intracellular calcium flux assay determined Piezo1 activity in phMEC (CD31+) and phUSMC (CD31−) cells. phMEC, phUSMC, and HEK293 Piezo1^{KO} cells were pretreated 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 minutes. 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 panel* - 10x bright field images of (top) phMEC, (middle) phUSMC, and (bottom) Piezo1^{KO}. *right panel* - Calbryte-induced fluorescence after 3 μM Yoda1 stimulation. 20x fluorescent images of (top) phMEC, (middle) phUSMC, and (bottom) Piezo1^{KO}. Data presented as ± SD.

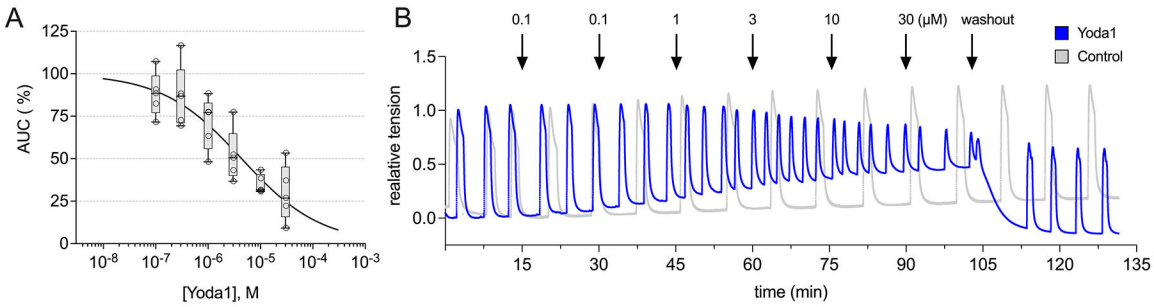

figure 4: EC_{50} of Piezo1 agonist (Yoda1) in human myometrium

A) TNL human myometrium (n=6) was hung in an organ bath, oxytocin-challenged (8nM), then dosed with the Piezo1 agonist, Yoda1, in 15 minute 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: 100nM (88.03%, SD 13.01), 300nM (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.

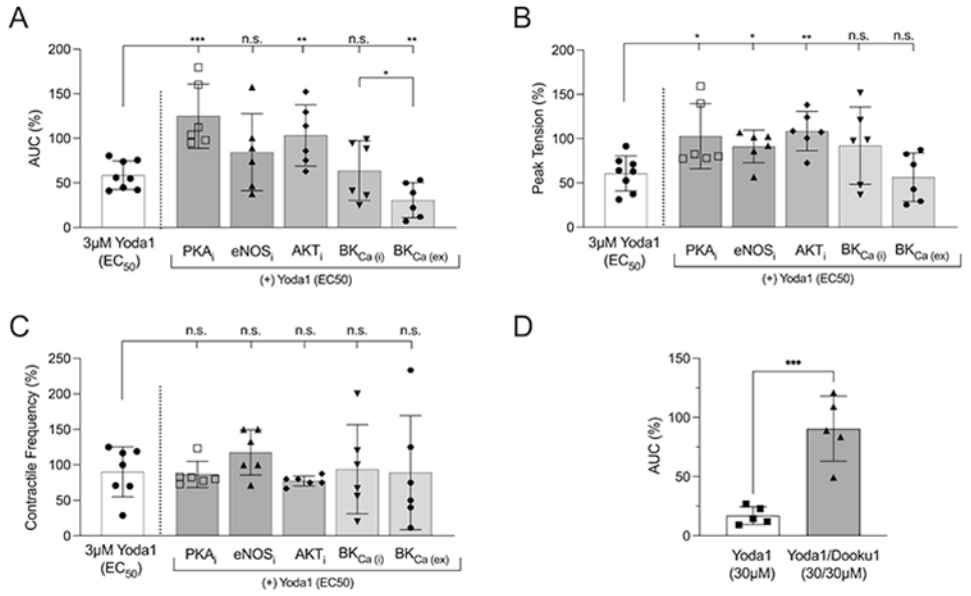

figure 5: Inotropic effects of Piezo1 agonism on Ca^{2+}-mediated myometrial quiescent pathways

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 (3μM) 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 deviated 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 30μM 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).

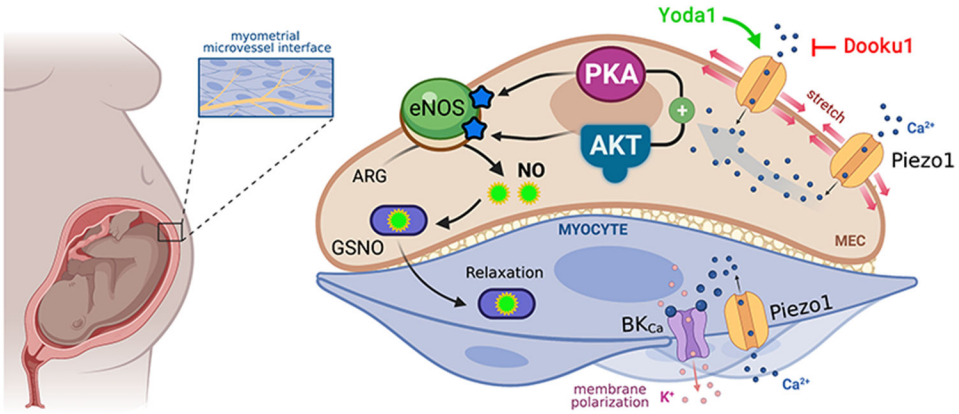

Figure 6: Proposed pathway for Piezo1-mediated quiescence in human myometrium

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.

Table 1:

Pregnancy Data

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 ± SD.

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