Expression of Stretch-Activated Two-Pore Potassium Channels in Human Myometrium in Pregnancy and Labor

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Abstract

Background: We tested the hypothesis that the stretch-activated, four-transmembrane domain, two pore potassium channels (K2P), TREK-1 and TRAAK are gestationally-regulated in human myometrium and contribute to uterine relaxation during pregnancy until labor.

Methodology: We determined the gene and protein expression of K2P channels in non-pregnant, pregnant term and preterm laboring myometrium. We employed both molecular biological and functional studies of K2P channels in myometrial samples taken from women undergoing cesarean delivery of a fetus.

Principal Findings: TREK-1, but not TREK-2, channels are expressed in human myometrium and significantly up-regulated during pregnancy. Down-regulation of TREK-1 message was seen by Q-PCR in laboring tissues consistent with a role for TREK-1 in maintaining uterine quiescence prior to labor. The TRAAK channel was unregulated in the same women. Blockade of stretch-activated channels with a channel non-specific tarantula toxin (GsMTx-4) or the more specific TREK-1 antagonist L-methionine ethyl ester altered contractile frequency in a dose-dependent manner in pregnant myometrium. Arachidonic acid treatment lowered contractile tension an effect blocked by fluphenazine. Functional studies are consistent with a role for TREK-1 in uterine quiescence.

Conclusions: We provide evidence supporting a role for TREK-1 in contributing to uterine quiescence during gestation and hypothesize that dysregulation of this mechanism may underlie certain cases of spontaneous pre-term birth.

Introduction

Premature birth is now the leading cause of newborn death worldwide [1] and comparable to the number of deaths from HIV/AIDS [2]. It accounts for 12% of all live births in the United States [3], 75% of all perinatal complications, the leading cause of fetal death [4] and is inexplicably more likely to occur in African American mothers [5]. Hospital charges for premature infants in the United States are estimated by the Institute of Medicine at $62 billion annually [7]. Despite improvements in prenatal, perinatal and neonatal care, the incidence of premature birth persists and is increasing [4]. To date, there is no effective means of treatment to prevent preterm delivery [8]. Indeed, until the development of the oxytocin receptor antagonist atosiban (Tractocile®, Ferring Pharmaceuticals), no treatment was developed specifically based on myometrial pharmacology, the introduction of the β2 adrenergic agonist ritodrine notwithstanding. Administration of 17-hydroxyprogesterone caproate (17P) has held promise in the early clinical trial setting but it does not improve outcomes in twin pregnancies [9], nor does it appear to be generally useful in the clinical setting [10] although certain groups of patients may benefit [11]. Since the trigger(s) for preterm labor are not exclusively the actions of oxytocin and the enhanced contractility of laboring human myometrium is not all blocked by atosiban [12], the drug has not offered a major therapeutic advance. Treatment of mothers with so-called tocolytics (MgSO4, terbutaline, nifedipine) is ineffective beyond 48 hours and not without consequence [13,14]. Indeed, the therapeutic focus of tocolytic use is providing time to employ steroid to mature the fetal lung rather than preventing delivery until term. It is imperative that we understand the unique physiological mechanisms underlying pregnancy and parturition at the biochemical and molecular level in order to discover new approaches to the prevention of preterm labor.

We previously reported that calcium-activated potassium channels (KCa) are differentially regulated during gestation and suggested these channels as putative nitrosylation targets [15,16].
Furthermore, recent studies from gastrointestinal (GI) smooth muscle have suggested that part of the hyperpolarizing effects of NO may be mediated by stretch-activated potassium (K2P) channels [17]. These mechanosensitive potassium channels are thought to help maintain relaxation of myocytes in visceral hollow organs by hyperpolarizing the membrane and have been found to regulate responses to nitricergic stimulation [10]. Although claimed to be absent in murine myometrium [10], we have described the expression of the potassium channels, subfamily K, member 4 (KCNK4) a two-pore potassium channel (K2P.4.1) known as TRAAK [TWIK-related acid-sensitive K⁺ channel] and member 2 (KCNK2) a two-pore potassium channel (K2P.2.1) known as TREK-1 [TWIK-related K⁺ channel] in human myometrium [19] as did Bai et al. in the same year [20]. Given these observations, we became interested in the regulation of expression and signaling of these K2P channels in human pregnancy myometrium in term and preterm labor. Since the uterus undergoes unprecedented expansion and stretch during gestation, we suggest that the stretch-activated K2P channels are involved in the maintenance of uterine quiescence prior to the onset of labor.

Stretch-activated K2P channels make up a unique subset of K⁺ channels that are mechano-sensitive and belong to a larger family of channels characterized by four transmembrane segments (TMS) and two pore (2P) domain regions. Unlike other members of the 4TMS/2P channel family TREK-1, TREK-2, and TRAAK belong to the TRAAK-family subset of K2P channels (KCNK2, KCNK10 and KCNK4) that are activated by arachidonic acid and increased membrane tension [21–23], both of which play a role during parturition [24,25]. These channels which are thought to form mature channels as homo- or heterodimers, are also known as leak or background K⁺ channels and play an essential role in setting the resting membrane potential of myocytes [23,26]. Examination of the literature reveals conflicting evidence for the expression of these K2P channels in uterine muscle [18,20,27,28]. Therefore, the expression and differential regulation of TRAAK-family channels during pregnancy and labor in human myometrium is of much interest, especially as they, or their regulation may represent potential therapeutic targets in pre-term labor.

Here we report that TRAAK-family members TREK-1 and TRAAK, but not TREK-2, are expressed in human pregnancy myometrium and that TREK-1 is differentially regulated during pregnancy. We provide evidence that inhibition of myometrial K2P channels (i.e., TREK-1) increases uterine excitability while activation of these channels lowers it. This work suggests that TRAAK-family channels, especially TREK-1, may be important in regulating uterine relaxation during pregnancy and hints at the possible dysregulation of this mechanism in pre-term births.

### Methods

#### Ethics Statement

The research presented here was reviewed and approved in writing by the University of Nevada Biomedical Review Committee (IRB) for the protection of human subjects in research.

#### Tissue Collection

With informed consent obtained in writing, samples of non-pregnant and pregnant (laboring and non-laboring) uterine tissue were obtained either via hysterectomy in pre-menopausal women ≤ 43 y undergoing hysterectomy when no uterine pathology is present, or elective cesarean section. Samples of non-pregnant uterine tissue were taken from the mid body following inspection by the pathologist, while samples from pregnant women were taken from the upper portion of the transverse uterine incision. Women were selected at random without inclusion criteria other than a clinical decision to deliver a pregnancy by Caesarian section (Table 1). Exclusion criteria were age less than 18 years, multiple pregnancy, known illicit drug use, or HIV or hepatitis C infection. Within 20 min of their removal, fresh tissue samples were transported to the laboratory in cold physiological buffer containing (in mM): NaCl (120), KCl (5), KH₂PO₄ (0.587), Na₂HPO₄ (0.589), MgCl₂ (2.5), Dextrose (20), CaCl₂ (2.5), Tris (25), and NaHCO₃ (5), adjusted to pH 7.4. Tissues collected for molecular biological studies were preserved and transported in RNAlater (Ambion, Austin, TX). Tissue collection was approved through the University of Nevada, Biomedical Institutional Review Board (IRB).

Uterine smooth muscle (myometrium) was first dissected from human uterine tissue samples and then either flash frozen in liquid nitrogen and stored at −80°C for later analysis or immediately utilized in contractile studies.

#### Semi-quantitative PCR

Total RNA was extracted from 50 mg (wet wt.) of myometrium in TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol and resuspended in 30 µl nuclease-free H₂O. DNA contamination was removed by treatment at 37°C with 10 U RNase-free DNase I (Promega, Madison, WI). DNase was inactivated by adding 25 mM EDTA with heating at 55°C for 10 min. cDNA was synthesized from 1 µg of total RNA using 250 ng random primers (Invitrogen), 0.125 mM each dNTPs, 10 mM DTT and 200 U Superscript II reverse transcriptase (Invitrogen).

Gene specific primers for human TRAAK, TREK-1, and TREK-2 (Table 2) were designed from areas of high homology between respective channel sequences from various published

### Table 1. Patient characteristics-pre-gravid tissues.

| Ethnicity | Diagnosis |
|-----------|-----------|
| Age (y) | White | Latino | A. Am. |
| Hysterectomy | (38–43) | 4 | 8 | 4 | Elective (all without disease) |

| Patient Characteristics-Gravid Tissues |
|-------------------------------------|
| Term (38–41 wks.) | (22–38) | 9 | 13 | 5 | Elective C-Section (all) |
| Term in Labor (39–40 wks.) | (26–32) | 4 | 7 | 2 | Elective (8) Breech (3), Placenta Previa (2) |
| Preterm in Labor (26–34 wks.) | (24–30) | 2 | 0 | 3 | PROM (2), Cervical Dilation >7 cm (3) |

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sequences using Integrated DNA Technologies Primer Quest software (Coralville, IA). Basic local alignment search tool (BLAST) searches were performed to confirm that primer sequences had no homology with any other known gene products. β-actin primers were designed to amplify both genomic (750 bp) as well as non-genomic products (500 bp) to control for genomic DNA contamination, while non-template controls ensured the integrity of the PCR reaction. Amplification was performed with the Quantum RNA 18S Internal Standards according to the manufacturer’s protocol (Ambion). An optimized ratio (1:20) of 18S rRNA primers was added to the reaction as an endogenous standard along with competitor to modulate 18S amplification without affecting the gene-specific PCR targets. PCR amplification within the linear range was carried out in a thermocycler under the following conditions: 95°C for 10 min as an initial melt, followed by 40 cycles of 95°C for 30 sec, annealing between 58°C to 60°C for 1 min, and extension at 72°C for 1 min; followed by a final extension of 10 min. We determined the linear range of our PCR reactions by increasing cycle number and resolving bands by electrophoresis. The products were visualized by ethidium bromide staining and quantitated by computer.

### Quantitative Real Time PCR

Human myometrial tissues were homogenized in TRIzol reagent, total RNA was isolated, and cDNA was synthesized as described above from 25 μg (wet wt.) of myometrium and diluted 1:5. TREK-1 QPCR was carried out using SYBR I green dye and TRAAK QPCR was carried out using Taqman gene expression assays (Table 2; Applied Biosystems, Foster City, CA), both using an ABI Prism 7000 sequence detection system.

Each SYBR green reaction (25 μl total) contained 2 μl cDNA for 18S, 10 μl cDNA for TREK-1, 12.5 μl SYBR Green PCR 2X Master Mix, and 400 nM forward and reverse primers. Samples were heated to 50°C for 2 min, melted at 95°C for 10 min, and then cycled 45 times at 95°C for 15 sec, followed by annealing and extension at 60°C for 1 min. A single final dissociation step included 95°C for 15 sec, 58°C for 20 sec, and 95°C for 15 sec. Amplification of the message was monitored by measuring the increase in fluorescence caused by SYBER I green binding to double-stranded DNA, resulting in an amplification plot of fluorescence vs. cycle number.

Each Taqman reaction (25 μl total) contained 2 μl cDNA for 18S, 8 μl cDNA for TRAAK, 12.5 μl Taqman Universal PCR 2X Master Mix, 900 nM forward and reverse primers, and 250 nM probe. Samples were heated to 50°C for 2 min, melted at 95°C for 10 min, and then cycled 45 times at 95°C for 15 sec and 60°C for 1 min. Amplification was monitored by measuring the increase in fluorescence caused by the 5’ to 3’ nuclease activity of the Amplitaq Gold enzyme cleaving the fluorescently-labeled probe, resulting in an amplification plot of fluorescence vs. cycle number.

For both methods, standard curves were generated for each target gene using serial dilutions of cDNA. The amount of specific target genes in unknown samples was calculated by measuring the cycle threshold (Ct) values and extrapolating starting copy numbers from standard curves. All samples were tested in triplicate and normalized to 18S rRNA amplified from respective samples to control for variations in sample quality. Non-template controls using water in place of cDNA were included in all QPCR plates to ensure the integrity of reaction components.

### Western Blotting

Flash frozen myometrial samples were homogenized and sonicated in buffer consisting of 1% (v/v) Triton X-100, 150 mM NaCl, 10 mM NaH₂PO₄, 5 mM EDTA, and 1X HalTAG Protease Inhibitor Cocktail (Pierce, Rockford, IL). This cell lysate was then centrifuged at 14,000 x g at 4°C for 30 min. The supernatant from each sample was then tested for protein concentration via Lowry assay (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA) as a standard.

Protein from lysate supernatants (30–40 μg) was boiled for 5 min in 1X denaturing sample loading buffer containing: 0.06 M Tris-HCl (pH 6.8), 10% glycerol (v/v), 2% SDS (w/v), 0.03% bromophenol blue (w/v), and 5% β-mercaptoethanol (v/v). Proteins were separated by electrophoresis in 10% polyacrylamide

### Table 2. PCR primers.

| Gene       | GenBank Accession No. | Semi-Quantitative PCR primer sequence | Product size (bp) |
|------------|-----------------------|---------------------------------------|-------------------|
| TRAAK      | AF247042              | F: 5'-TCTCAAGCCGTGTTGGCTGCTCT-3' R: 5'-ATTGATCGGAGCTTGGGACACAG-3' | 240               |
| TREK-1     | NM_001017424          | F: 5'-TGCGTGTAGCTTGGGCTGCTCT-3' R: 5'-ACTTAGCTGCCCTTGGTTATTCCTTT-3' | 349               |
| TREK-2     | NM_021161             | F: 5'-TGTTGGGCTGCACACTTGGCAG-3' R: 5'-ACACACACACACACACACACACACACACCC-3' | 756               |

| Gene       | GenBank Accession No. | Quantitative PCR primer/probe sequence | Product size (bp) |
|------------|-----------------------|---------------------------------------|-------------------|
| TREK-1     | AF129399              | F: GGA ATT CCC CTC TTT GGT TTT C R: CAC TTT GGG AAT TCC TTT TCC A | 82                |
| 18S        | X03205.1              | F: CAC GGC CGG TAC AGT GAA A R: AGA GGA GCG AGC GAC CAA | 72                |
| TRAAK      | NM_033310.2           | TaqMan Gene Expression Assay Hs99999901_s1, Applied Biosystems | 187               |
| 18S        | X03205.1              | TaqManGene Expression Assay Hs00213267_m1, Applied Biosystems | 101               |

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gels (Bio-Rad, Hercules, CA) and transferred to a nitrocellulose membrane. Membranes were blocked at 4°C overnight in a 1:1 solution of Odyssey™ blocking buffer (Licor Biosciences, Lincoln, NE) and phosphate buffered saline [PBS; 137 mM NaCl, 2.7 mM KCl, 0.9 mM KH₂PO₄, 6.4 mM Na₂HPO₄, adjusted to pH 7.4]. The membranes were then labeled for either human TREK-1 (1:1000 rabbit IgG; Santa Cruz Biotechnology, CA) or TRAAK (1:1000 goat IgG; Santa Cruz Biotechnology). Respective secondary antibodies conjugated to either infrared 680 or infrared 800 fluorescent dye (1:100,000; Invitrogen or Rockland Immunocyticals, Philadelphia, PA) were used for detection. Antibody incubations were carried out in 1:1 Odyssey™ blocking buffer (Licor Biosciences) and PBS with 0.1% Tween-20 (v/v) at 4°C. Bands were visualized using an infrared imaging system (LI-COR Biosciences) and PBS with 0.1% Tween-20 (v/v) at 4°C.

Results

TREK-1 Gene and Protein Expression in Human Myometrium

Relative myometrial TREK-1 mRNA expression was determined by PCR and normalized to β-actin expression (Fig. 1A). TREK-1 primers (Table 2) gave a 240 base pair transcript found to be expressed in both pregnant and non-pregnant human myometrial smooth muscle (Fig. 1B) at similar levels. Human brain was included as a positive control for TREK-1 expression. The relative quantification of TREK-1 TREK-1 primer amplicons was determined using the comparative CT method ([ΔΔCT]) and normalized to GAPDH (1:1500 mouse IgG; Santa Cruz Biotechnology). Control experiments were developed for GAPDH as a loading control and to normalize the TREK-1 antibody. Samples were run in triplicate and statistical analyses were performed using GraphPad software. Statistical significance was determined using a Student t-test (GraphPad Software, San Diego, CA) with p<0.05 considered significant.

TREK-1 protein expression in myometrial samples was determined using Western blot (Fig. 2A). Control experiments using TREK-1 over expressing COS cells demonstrated that the TREK-1 antibody ([Santa Cruz, CA]) was specific for TREK-1 expression (data not shown). TREK-1 antibody revealed a single band at 47 kDa (under reducing conditions) and immunoblotting were developed for GAPDH as a loading control and to normalize TREK-1 expression (Fig. 2A). No significant difference was found in the expression of TREK-1 in 17 term pregnant samples (38-40 weeks gestation) compared to 16 samples from non-pregnant myometrium [15]. A difference in TREK-1 expression in laboring tissues taken from 13 women at term (39-40 weeks gestation) versus non-laboring tissues taken from 27 women at term (38-41 weeks gestation) was not significant. Power analysis confirmed that we would measure a difference if one existed at this level with 99% power to detect a significant difference at p<0.05.

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TREK-1 Gene and Protein Expression

TREK-1 mRNA was expressed in both non-pregnant and pregnant human myometrial smooth muscle (Fig. 1A). Amplification of TREK-2 message from either non-pregnant or pregnant human myometrium yielded no evidence of expression consistent with the findings of Bai, et al. [20]. Ribosomal 18S RNA was used to normalize PCR reactions [15]. Expression of TREK-1 in
isolated myometrial cells confirmed the origin of these channels as myocyte proteins (Fig 3A). TREK-1 mRNA expression varied significantly between non-pregnant and pregnant myometrium, demonstrating a 2-fold up-regulation during pregnancy toward term (70.2 ± 134.2%; p < 0.01; Fig. 3B) suggesting the possibility that TREK-1 plays a role during pregnancy. In order to determine the effect of labor at term, TREK-1 gene expression was examined in these patient samples by Q-PCR. The elevated expression seen in pregnant term samples when compared to laboring term samples revealed a dramatic decrease in gene expression consistent with the notion that TREK-1 contributes to myometrial quiescence at term (Fig. 3C). Because the absence of TREK-1 could contribute to preterm labor, we also determined TREK-1 expression in samples from women in labor at 28–33 weeks gestation. TREK-1 gene expression in preterm myometrium (Fig. 3C) was comparable to expression in laboring samples.
Translation of human myometrial TREK-1 gene into protein was confirmed in both pregnant and non-pregnant tissue samples (Fig. 4A) by Western blot quantified for both TREK-1 (48 kDa under reducing conditions) and GAPDH. TREK-1 protein expression showed a significant increase during pregnancy (26%, Fig. 4A) consistent with gene expression data (Fig. 3B). At the time of labor however, TREK protein expression measured by Western blot is still present at pre-laboring levels but is significantly lower in preterm samples (Fig. 4B).
Inhibition of Stretch-Activated Channels Stimulates Myometrial Contractions

To verify the significance of stretch-activated channels to the physiological function of the myometrium, we explored the effects of inhibiting stretch-activated channels. In order to test the hypothesis that stretch-activated channels assist in the regulation of phasic contractions in myometrial smooth muscle, we utilized contractile bath studies. Increasing concentrations of GsMTx-4 (Grammostola spatulata mechanotoxin-4), a specific mechanosensitive channel blocker [35], amplified oxytocin (1 μM; OT) induced contractions in non-laboring pregnant myometrium. Addition of GsMTx-4 at 0.9 μM caused an increase in duration of contractions (Fig. 5A), while higher concentrations such as 1.8 μM toxin caused both an increase in duration and frequency of contractions (Fig. 5B). This effect was not seen on spontaneously contracting tissue strips (control, no OT treatment) and the degree of effect varied greatly from patient to patient but was consistently seen (data not shown).

Because GsMTx-4 affects all stretch-activated channels and thus in myometrium blocks both TREK-1 and TRAAK, we further tested the hypothesis that a TREK-1 more specific stretch-activated potassium channel inhibitor would diminish the ability of the myometrium to relax. It has been shown in bladder smooth muscle that methionine and its derivatives inhibit TREK channels, thereby increasing bladder excitability [17,33]. We observed that increasing concentrations of L-methionine ethyl ester (L-Mec) augmented spontaneous contractions in non-laboring myometrium. Addition of L-Mec at 300 μM caused an increase in frequency of contractions (Fig. 6). We further normalized each tissue to itself under control conditions (100 nM iberiotoxin) and observed that L-Mec increased myometrial frequency of contraction in a dose dependent manner (Fig. 6B). L-Mec significantly inhibited myometrial relaxation (i.e. increased contractions) at concentrations ≥100 μM (p<0.05) and reached maximal effect at ~300 μM where contractile frequency was increased to ~35% above control levels (Fig. 6B). At concentrations of L-Mec ≥1 mM we observed a reduction in peak contraction amplitude and a diminished ability for tissue recovery after washout of drug; tissues treated with 10 mM L-Mec failed to contract or relax (data not shown).

Fluphenazine and other antipsychotics have also been described as selective K2P blockers able to inhibit TREK-1 but not TRAAK channels [34]. We conducted contractile bath experiments in pregnant tissues using the K2P channel activator arachidonic acid (AA; 10 μM) and the putative TREK-1 inhibitor fluphenazine (FLU; 100 μM). OT (100 nM) stimulated contractions (ex. Fig. 6A) were blunted by the addition of AA compared to control consistent with TREK-1 channel activation (Fig. 6C). Addition of fluphenazine alone did not significantly alter contractility, while addition of FLU to oxytocin treated tissues in the presence of AA prevented the diminished contractility seen with AA alone (Fig. 6C). Tissues responded to OT stimulations in a fashion comparable to control after washout of AA and FLU (Fig. 6C. Post-Con). Concentrations of FLU greater than 300 μM suppressed OT-induced contractions and were irreversible during the course of the experiment suggesting non-specific effects (not shown).

Discussion

While many factors are thought to modulate the contraction and relaxation of uterine smooth muscle, it is generally agreed that membrane ion channels are crucial to this process and are likely targets of many of the factors which regulate myometrial tone. Interest in stretch-activated channels in the myometrium stems from the knowledge that the uterus enlarges to accommodate a growing fetus during pregnancy. Evidence supports the general notion that potassium channels maintain the uterus in a quiescent state during gestation [36]. We further hypothesized that stretch activated potassium channels are differentially regulated and contribute substantially to the resting membrane potential in the pregnant myometrium, as well as serving to counteract contractile stimuli. We were therefore interested in elucidating the role and possible regulation of the stretch-activated channels, TREK and TRAAK. These channels and the factors that regulate them may provide a unique therapeutic target to regulate the contractility of the myometrium in cases such as preterm labor.

The stretch-activated channel TREK-1 was previously reported to only be minimally expressed in human myometrium [20,28,37], while the K2P channels TREK-2 and TRAAK were thought not to be expressed in this tissue at all [18,20,38]. Here we report that TREK-1 and TRAAK, but not TREK-2, transcripts and protein are expressed in human myometrium. Furthermore, TREK-1 but not TRAAK is substantially up-regulated in pregnant samples when compared to non-pregnant samples consistent with a functional role for TREK-1 channels in pregnancy. The stretch-activated channel toxin (GsM Tx-4) and the TREK-1 more selective channel inhibitors methionine ethyl ester and fluphenazine altered myometrial tension and contractile frequency...
consistent with the activity of TREK-1 currents in pregnancy myometrium. Increased TREK-1 channel expression as well as function in pregnant myometrium suggests that as pregnancy progresses and the stretch of the uterus increases, these K2P channels are activated to assist in the maintenance of relaxation. To the best of our knowledge, ours is the first work documenting the expression and action of TREK in the myometrium during human pregnancy and labor. This report of a regulated increase in both expression as well as channel function supports our hypothesis that TREK-1 channels are important in maintaining quiescence during pregnancy and that channel expression and or activation may be dysregulated in spontaneous pre-term labor.

This notion of differential expression of TREK-1 channels and their regulation of membrane excitability in normal physiology is supported by several reports in animal models. Differential regulation of TREK-1 has been shown in epicardial vs. endocardial myocytes in rat ventricle [39]. TREK-1 has further been observed to be developmentally regulated in rat ventricle...
with a suggested role in reducing cardiac excitability due to its hyperpolarizing effect [40]. Evidence also suggests the regulation of K2P channel expression and function in pathophysiological states. Elevated levels of TREK-1 mRNA and protein have been seen in hypertrophic myocardium [41]. TREK-1 and TRAAK have also been shown to be up-regulated in a rat model of experimental acute cerebral ischemia [42].

It is known that ischemia can activate phospholipase A2 and result in the accumulation of unsaturated fatty acids such as arachidonic acid. Accumulation of arachidonic acid would lead to activation of TREK, thereby causing an efflux of K+ ions and allow for membrane hyperpolarization to decrease cell excitability. This mechanism is hypothesized to provide a protective effect during cerebral ischemia and may parallel the mechanism by which we hypothesize the up-regulation of myometrial TREK-1 channels during gestation to help maintain the uterus in a relative state of quiescence. Blockade of stretch-activated channels by GsMTx-4 in pregnant human myometrium increased contractions and subsequently attenuated normal relaxation mechanisms in non-laboring tissues. Stretch-activated K+ channels are likely to be significant contributors to the relaxed state in pregnancy because activation by arachidonic acid lowers myometrial tension in oxytocin-stimulated tissues, an effect blocked by fluphenazine while fluphenazine alone had no effect in our studies. Inhibition by L-Mec increases contractile frequency in non-laboring tissues consistent with removal of a relaxation influence on the muscle. Our findings are consistent with previous studies showing the inhibition of TREK-1 channels by methionine containing compounds or fluphenazine [17,33,34]. The mechanism of TREK-1 activation and inhibition in human myometrium is unknown and thus the failure of fluphenazine to alter contractions when added alone and yet block an effect of arachidonic acid are unexplained. These results may reflect distinctions in the manner of channel inhibition by these agents. There is a clear dissociation between TREK-1 gene expression and protein expression in the samples tested (Fig. 3C vs. 4B). If our thesis is correct, this result may not reveal a distinction between gene regulation, reduced at the time of labor, and the presence of protein detectable in Western blots. While it is entirely likely that the time needed to see a fall in protein following changes in gene expression may not be provided for in laboring tissues, it is also possible that we do not detect labor-associated regulation of channel function such that continued presence of the protein is not inconsistent with labor. The decreased expression of both message and protein in preterm tissues however (Fig. 3C, 4B) is consistent with our hypothesis. Potassium channel internalization (a state of channel not reflected by gene or protein expression), has been shown to be affected by post-translational modifications which can be differentially regulated depending on metabolic states [43–45]. In addition to regulation of K2P channels via expression levels and post-translational modification, reports also support the presence of splice variants with differing channel activity. Alternative splicing of potassium channels has previously been shown to contribute to the diversity of channel specificity in myometrium [36,46–48], as well as many other tissues [49–52]. More importantly, TREK-1 has been shown to be alternatively spliced in rat heart suggesting two different channel isoforms; one of lower (~41 pS) and one of higher (~132 pS) conductance, both stimulated at positive potentials [53]. This information, as well as a survey of available TREK-1 sequence variants on GenBank, leads us to hypothesize alternative splicing of TREK-1 in different states of the uterus; a dysregulation or shift in predominant variant(s) and or assembly of channels as homo- or heterodimers in a regulated fashion by pregnancy may explain TREK-1 activation in pregnancy myometrium and alterations in this process may predispose certain women to spontaneous pre-term labor contributing to pre-term birth.

Difference in the expression of TREK-1 seen in pregnant versus non-pregnant myometrium are not thought to be the result of the age difference in patients in these two groups (Table 1). The fact that TRAAK expression was not different argues in favor of age not being a factor. However, age is significantly different between pre-gravid and gravid women but does not represent power sufficient to support a comparison of age difference in each pregnancy group versus pre-gravid women. We do not think it likely that age per se, influences our results.

In summary, our results provide strong evidence supporting the hypothesis that the K2P channel TREK-1 is functionally up-regulated in pregnancy, and differentially regulated during pregnancy to relax the uterus prior to labor. We hypothesize that TREK-1 is functionally dysregulated in spontaneous pre-term birth, contributing to the disruption of normal myometrial quiescence during pregnancy. This dysregulation may be the result of changes in channel expression levels, post-translational modulation and/or variant channel expression and dysfunctional channel assembly. TREK-1 channels and or their regulation offer promise as potential therapeutic targets in controlling pre-term uterine contractions.

Author Contributions
Conceived and designed the experiments: ILOB CAS JNT. Performed the experiments: JNT. Analyzed the data: ILOB CAS JNT. Contributed reagents/materials/analysis tools: CAS. Wrote the paper: ILOB.

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