The role of the RHOA/ROCK pathway in the regulation of myometrial stages throughout pregnancy

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BACKGROUND: Controlling uterine contractile activity is essential to regulate the duration of pregnancy. During most of the pregnancy, the uterus does not contract (i.e., myometrial quiescence). The myometrium recovers its contractile phenotype at around 36 weeks (i.e., myometrial activation) through several mechanisms. The RHOA/ROCK pathway plays a vital role in facilitating muscular contractions by calcium sensitization in humans. Yet, the role of this pathway during different myometrial stages, including quiescence, has not been elucidated.

OBJECTIVE: we aimed to study the role of the RHOA/ROCK pathway in the regulation of the different myometrial stages throughout pregnancy. Specifically, we hypothesized that the inhibition of the components of the RHOA/ROCK pathway play an important role in maintaining uterine quiescence.

STUDY DESIGN: Myometrial samples were obtained from pregnant individuals who underwent cesarean section. Pregnant individuals who delivered preterm without labor (myometrial quiescence), preterm with labor (nonphysiological myometrial stimulation), term not in labor (activation), and term in labor (physiological myometrial stimulation) were included. The mRNA and protein expression of RHOA, ROCK I, ROCK II, RND1-3, and ROCK activity through pMYTP1 were evaluated.

RESULTS: We found that the human myometrium constitutively expressed RHOA/ROCK pathway components throughout pregnancy. No changes in the components of the RHOA/ROCK pathway were found during quiescence. Moreover, the RHOA protein and ROCK activity increased in the myometrium during labor, supporting the hypothesis that this pathway participates in maintaining the contractile activity of the myometrium. This study provides insight into the role of the RHOA/ROCK pathway in controlling myometrial contractile activity during pregnancy.

Key words: labor, myometrium, myometrial activation, myometrial quiescence, uterine contraction

Introduction

Labor is a complex and well-orchestrated process that involves uterine contractions, decidual/chorioamniotic membrane activation, and cervical effacement/dilatation, collectively known as the common pathway of parturition. Each component of this pathway is triggered by multiple factors, including physiologic inflammation as well as maternal and fetal endocrine signals, such as functional progesterone withdrawal.[1–4] The coordination and timing of these factors during gestation are essential for a successful physiological parturition. Multiple investigations have focused on deciphering the clock that allows the timely coordination of this cascade of events.[4–7] Indeed, the untimely or uncoordinated activation of the common pathway of parturition can result in pathological outcomes, such as preterm births or post-term deliveries. While all three components of the common pathway of labor are essential, the most striking event is uterine contraction. The uterus is essentially a contractile organ; with the myometrium serving as its functional unit. The myometrium undergoes four stages of contractile activity throughout gestation: quiescence; activation, stimulation, and involution.[1,3,8–10] Myometrial quiescence is a period of active relaxation that starts after embryo implantation and continues until around 36 weeks, leading to myometrial activation, recovering its contractile phenotype.[1,3,8–10] Following activation, myometrial stimulation begins, characterized by coordinated uterine contractions that culminate with the delivery of the fetus, eventually leading to myometrial involution.[1,3,8–10] The myometrial activation is mediated in part by reduced progesterone action, leading to increased expression of contraction-associated proteins (CAPs).[3,10–12]

Multiple factors are involved in uterine quiescence and activation.[1,3,8–10] Interestingly, the RHOA/ROCK pathway has been shown to play a key role in facilitating myometrial contractions through calcium sensitization in humans.[13–15] RHO proteins belong to the small monomeric G proteins' family, from the Ras superfamily of GTPases.[16,17] RHO GTPases are found in all eukaryotic organisms and fulfill multiple functions.[18] RHO proteins' main intracellular effector is ROCK, which has two isoforms, ROCK1 and ROCK2.[19,20] The RND proteins are part of the RHO family and inhibit the

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Why was this study conducted?

This study was conducted to investigate the role of the RHOA/ROCK pathway in the regulation of different myometrial stages throughout pregnancy, specifically focusing on whether inhibition of this pathway is crucial for maintaining uterine quiescence. The understanding of these mechanisms could provide valuable insights for developing therapeutic strategies to manage labor abnormalities such as preterm birth.

Key findings

The study found that the RHOA/ROCK pathway is constitutively expressed in the human myometrium throughout pregnancy, with no significant changes in its components during quiescence. However, the protein expression of RHOA and ROCK activity significantly increased during labor. These results suggest that while the RHOA/ROCK pathway does not drive myometrial quiescence, it plays an important role in promoting the contractile phenotype of the myometrium during labor.

What does this add to what is known?

This study adds new insights into the temporal regulation of the RHOA/ROCK pathway during pregnancy. It refutes the hypothesis that inhibition of this pathway by RND proteins is necessary for maintaining myometrial quiescence and instead highlights the pathway's involvement in the activation of uterine contractions during labor. This understanding could aid in the development of novel therapeutic approaches for the prevention of preterm labor.

RHOA/ROCK pathway.[21–23] When RND binds to the RHOA/ROCK complex, it inhibits the calcium sensitization mediated by RHOA.[21,23] A previous study suggested that RND could reduce the myometrial contractions during pregnancy due to the increased concentration of RND2 and RND3 in myometrial samples of pregnant compared to nonpregnant women.[24] Yet, whether RND proteins inhibits RHOA/ROCK activation during quiescence has not been investigated.

Therefore, we hypothesize that the inhibition of components of the RHOA/ROCK pathway play an important role in maintaining uterine quiescence. To test this hypothesis, we aim to study the RHO family (RHOA and RND proteins) and ROCK in human myometrium across gestation to determine this pathway's role in the myometrial quiescence/activation/stimulation cycle.

Materials and methods

Ethical approval

The Institutional Review Board at the Pontificia Universidad Católica de Chile, where all samples were obtained, approved the study protocol (#13-217). Women were included only after providing informed consent.

Patients and tissue samples

Clinical and demographic characteristics are shown in Table 1. Cesarean sections were indicated for medical reasons independent of this study. Myometrial samples were obtained from 4 groups of pregnant individuals: preterm not in labor (PT-NL), preterm in labor (PT-L), term not in labor (T-NL), and term in labor (T-L). Labor was diagnosed by regular uterine contractions (≥2 contractions every 10 minutes) associated with cervical changes (≥80% effacement and ≥2 cm dilation). Preterm samples were taken from women who delivered between 30 and 34 weeks of pregnancy, and term samples were taken from women who delivered between 38 and 40 weeks. Given that myometrial activation typically begins at 36 weeks of gestation, patients <34 weeks or >38 weeks were included, to ensure myometrial quiescence and activation,

TABLE 1
Characteristics of study participants

	PT-NL (*n*=8)	PT-L (*n*=8)	T-NL (*n*=8)	T-L (*n*=8)
Maternal age	33.4±4.1	32.9±6.5	30.4±5.5	33.2±4.9
Body mass index	26.8±5.12	27.1±5.43	29.8±7.62	29.5±8.23
Gestational age at delivery	30 (30–34)	31 (30–34)	39 (38–40)	39 (38–40)
Birth weight	1436±657	1503±487	3465.0±719	3540.4±577
Cause of cesarean section	FGR *n*=4 Severe PE *n*=3 3rd Trimester bleeding=1	Non vertex=5 NRFS *n*=3	1 prior cesarean section=5 2 prior cesarean section=3	Protracted labor *n*=7 Non vertex=1

Preterm not in labor (PT-NL), preterm in labor (PT-L), term not in labor (T-NL), and term in labor (T-L). FGR = fetal growth restriction; Severe PE = preeclampsia with severe features; NRFS = nonreassuring fetal status.

Maternal age (years), body mass index, and birthweight (grams) data are expressed as mean±SEM; or median (range).

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TABLE 2
Primer pairs used for RT-PCR

Gen		Secuencia	Tm (°C)	Amplicon length (bp)
RHOA	Sense	5′-GACAGCCCTGATAGTTTAGAAAACA-3′	54,9	183
	Antisense	5′-CATATCTCTGCCTTCTTCAGGTTT-3′	54,7	
ROCKI	Sense	5′-CTCAAGACATGCTTAATCACTCAGA-3′	54,7	160
	Antisense	5′-GCCTCTTCAATAGATTGATGTTTGT-3′	53,6	
ROCKII	Sense	5′-AAGCCATCCTTCTAATCTTGATTCT-3′	56,2	150
	Antisense	5′-AAGCCATCCTTCTAATCTTGATTCT-3′	54,1	
RND1	Sense	5′-GTCGCTCTGAACTCATCTCTTCTAC-3′	56,5	166
	Antisense	5′-ACTCATGTCCAGTGTCCTAAATTGT-3′	56,2	
RND2	Sense	5′-AGTTATGTCCCCACCGTGTTT-3′	56,5	258
	Antisense	5′-TTTACAGCCAACCAGCACAA-3′	55,2	
RND3	Sense	5′-CTATGACCAGGGGGCAAATA-3′	54,2	245
	Antisense	5′-TCTTCGCTTTGTCCTTTCGT-3′	54,5	
GAPDH	Sense	5′-CCCCTGGCCAAGGTCATCCAT-3′	55,2	680
	Antisense	5′-CCCCTCCCCTCTTAAGGGGT-3′	55,5	

The primers were designed based on published complementary DNA (cDNA) nucleotide sequences using primer3.

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respectively. Patients with preterm labor and concurrent clinical or laboratory signs of infection were excluded. All women included in the study were Hispanic. A piece of 1 × 3 cm the superior edge of the hysterotomy was resected, which is equivalent to 5−10 gr of tissue. This section was performed after removal of the infant and removal of the placenta, prior to uterine closure. The samples were frozen immediately in liquid nitrogen, and stored at −80°C until use.

The preterm not in labor (PT-NL) myometrium represents myometrial quiescence. Term not in labor (T-NL) corresponds to myometrial activation, while term in labor (T-L) links to myometrial stimulation. The preterm in labor (PT-L) samples belongs to a non-physiological stimulation stage.[2]

RT-PCR

Reverse-transcription-PCR (RT-PCR) quantified messenger RNA for RHOA, RND1-3, and ROCK I-II from the myometrial samples. Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform extraction method.[25] The cDNA was synthesized from total RNA using the Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The reverse-transcribed cDNA was amplified in a 25-uL PCR reaction containing 200 mM dNTPs, 1 mM of each primer, 100 ng cDNA, 2.5 U Taq DNA polymerase (Invitrogen), and 2 mM/L MgCl2. The primers for RhoA, ROCK I, ROCK II, RND1, RND2 y RND3 were designed using Primer3 based on published cDNA nucleotide sequences[26] (Table 2). The cDNA was amplified using an initial denaturation step at 94°C for 5 minutes, followed by a cycling stage with a specific number of cycles determined by a cycle curve developed for each gene (94°C for 30 seconds for denaturation, annealing or primer binding (Tm-5°C) for 30 seconds, and elongation at 72°C for 30 seconds). The resulting PCR products were subjected to electrophoresis on a 1.5% agarose gel with fluorescent staining (GelRed nucleic acid gel stain, Biotium, Fremont, CA, USA) (1 μL/10 mL), run at 80V in 1X TAE Running Buffer (Winkler, Santiago, Chile), and the bands were visualized by UV transillumination. A DNA molecular weight marker (100-bp DNA ladder; Winkler) was run alongside the samples to identify the size of the desired bands. The PCR bands were scanned, and the optical density of the bands was calculated. The arbitrary units of each band's optical density were normalized by the optical density of the GADPH mRNA that was used as an internal control for each PCR.

Western blot

Protein expression of RHOA, RND1-3, and pMYPT1 was measured by western blot. *Total protein extraction:* Approximately 200 mg of frozen myometrial tissue, stored at -80°C, was ground in a porcelain mortar with liquid nitrogen and then homogenized on ice with 500 μL of John's Lysis Buffer (20 mM Tris, pH 7.4, 1% Triton, 10% glycerol, 137 mM NaCl, 2 mM EDTA) supplemented with a protease inhibitor cocktail (1X) 40 μL/mL (cOmplete Mini, Roche diagnostics) and a phosphatase inhibitor 5 μL/mL (sodium orthovanadate 0.05 mM). The samples were incubated on ice for 30 minutes, vortexed for 20 seconds every 10 minutes. Subsequently, they were sonicated and centrifuged at 20,800 g (14,000 rpm) at 4°C for 5 minutes. The supernatant was then collected and frozen at -80°C. The total protein concentration was determined using the Bradford method, with the Bio-Rad Protein Assay reagent.

RhoA, Rnd1, and Rnd3: The membranes were blocked with 5% BSA in 1X TBS-Tween 0.1% for 1 hour at room temperature. They were then incubated overnight at 4°C with the following primary antibodies: anti-RhoA (67B9 Rabbit mAb, Cell Signaling, Danvers, MA, USA) anti-Rnd1 (Millipore, Darmstadt, Germany), and anti-Rnd3 (Millipore). The membranes were then washed three times with 1X TBS-Tween 0.1% using the SNAP i.d.™ system (Millipore) and incubated for 15 minutes with the secondary antibodies: anti-rabbit HRP (Rockland, Limerick, PA, USA) for RhoA and anti-mouse HRP (Chemicon, Rolling Meadows, IL, USA)

for Rnd1-3, both at a 1:1000 dilution in 0.5% BSA in TBS-Tween 0.1%. Three washes with TBS-Tween 0.1% were performed afterward.

Rnd2: Briefly, samples of the myometrium (50 μg of protein) were boiled (5 min, 90°C) in 6X loading buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 10% 2-bercaptoethanol), separated by SDS-PAGE (10% polyacrylamide gel), and electro-transferred to nitrocellulose membranes. The nitrocellulose membranes were blocked with 5% nonfat milk in TBS-T (2 hours at room temperature). The membranes were incubated with a primary anti-RND2 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for 1.5 hours at room temperature (1:1000 dilution). The membranes were washed 4 times with TBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit antibody, diluted 1:3000 in TBS-T (Millipore, Billerica, MA, USA), for 1 hour at room temperature. After four washes of 15 minutes each with TBS-T, immunoreactive bands were detected using the Western Lightning-ECL Plus method (PerkinElmer Inc., Waltham, MA, USA). The loading control was achieved by normalizing to β-actin. The optical density of the bands was measured for quantitative analysis.

p-MYPT1 (Thr853): The membranes were blocked with 7% nonfat dry milk in PBS-Tween 0.1% for 1 hour at room temperature. They were then incubated with the primary antibody anti-p-MYPT1 (Thr853) (1:1000) (sc17432, Santa Cruz) overnight at 4°C. Four 15-minute washes with PBS-Tween 0.1% were performed, followed by incubation with the secondary antibody anti-goat HRP (Chemicon) (1:3000) for 1.5 hours at room temperature. The membranes were then washed four times with PBS-Tween 0.1% for 15 minutes each. Total MYPT1: The membrane was stripped using a REBLOT Plus 1X solution (Millipore) for 15 minutes at room temperature with agitation. This was followed by two 8-minute washes with PBS-Tween 0.1%. The membrane was then blocked with 7% nonfat dry milk in PBS-Tween 0.1% for 1 hour at room temperature. The membrane was incubated with the primary antibody anti-MYPT1 (sc25618, Santa Cruz) (1:1000) overnight at 4°C. Four 15-minute washes with PBS-Tween 0.1% were performed. The membrane was then incubated with the secondary antibody anti-rabbit HRP (Rockland) (1:3000) for 1 hour at room temperature, followed by four 15-minute washes with PBS-Tween 0.1%.

β-actin: To verify correct loading and quantification, all membranes were probed for the constitutive protein β-actin, used as a control, following the western blot for the protein of interest. The membrane was incubated with the primary antibody anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA) (1:10000) in 5% BSA in TBS-Tween 0.1% for 1 hour at room temperature. This was followed by four 15-minute washes with TBS-Tween 0.1%. The membrane was then incubated with the secondary antibody anti-mouse HRP (Chemicon) (1:3000) for 1 hour at room temperature, followed by four 15-minute washes with TBS-Tween 0.1%.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism version 8.0.1 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The sample size has been established by our group to detect a 25% increase in mRNA or protein levels among the groups with 80% power and 5% alpha error, with 5 patients in each group.[27] Therefore, to ensure statistical power, we have included at least six samples in each determination. The numerical averages were calculated for each group and compared to the preterm not-in-labor group (PT-NL). The results are expressed as such in the figures.

All data sets were subjected to a test of normality (Shapiro−Wilk test), and parametric or nonparametric tests were performed when appropriate. Statistical comparisons among multiple groups were conducted using a one-way analysis of variance (ANOVA) test followed by posthoc Student-Newman-Keuls (parametric) or a Kruskall−Wallis One-Way ANOVA on Ranks test followed by Dunn's multiple comparison tests (nonparametric). A two-tailed $P<.05$ was indicative of a statistically significant difference.

Results

Expression of myometrial RHOA

RHOA mRNA was constitutively expressed in the myometrium throughout pregnancy (Figure 1B). The RHOA mRNA's relative expression in the myometrium from quiescence (PT-NL) did not differ from those of activation or stimulation (Figure 1B). RHOA protein's relative expression was higher in the myometrium from women with preterm and term labor than myometrium from women who delivered preterm without labor (Figure 1C). Interestingly, the protein expression of RHOA in myometrium from women at term without labor (activation) is not significantly different from that of preterm not in labor (quiescence).

Expression of myometrial ROCK

ROCK I mRNA was constitutively expressed across pregnancy; however, no differences were found between myometrial stages (Figure 2A). Similarly, ROCK II mRNA was also constitutively expressed throughout pregnancy; yet, its levels did not differ according to myometrial status (Figure 2B).

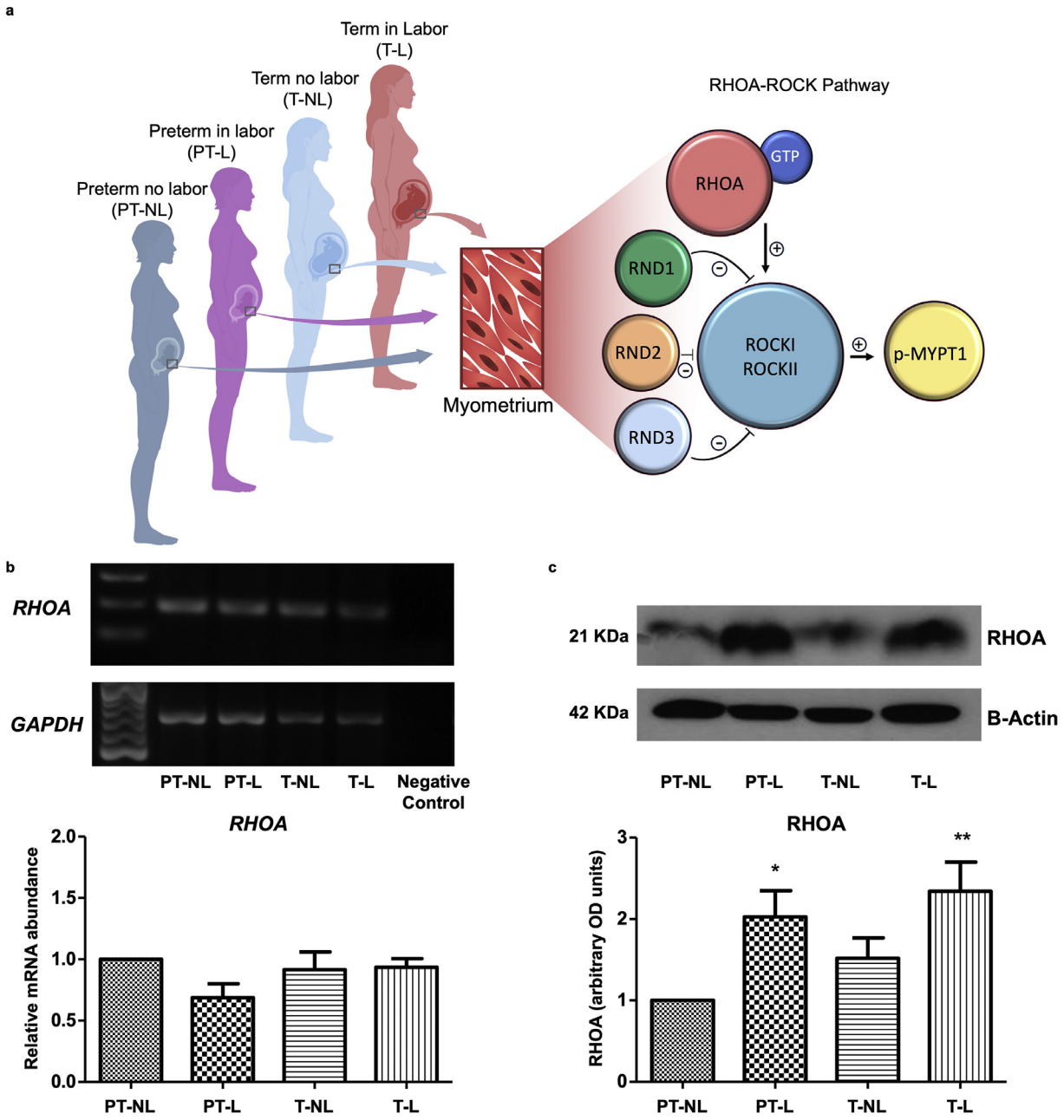
Expression of myometrial RND proteins

No changes were found in both the mRNA and the protein expression of RND1 (Figure 3A, B). The mRNA expression of RND2 was significantly lower in women who delivered at term without labor (activation) compared to women who delivered preterm without labor (quiescence) (Figure 3C). However, no differences were found in the protein levels of RND2 among groups (Figure 3D). Furthermore, there were no differences in the mRNA and protein expression of RND3 among the study groups (Figure 3E, F).

ROCK activity during pregnancy

The activated ROCK phosphorylates the regulatory subunit of the MLCP (MYPT1), facilitating myometrial

FIGURE 1
Expression of myometrial RHOA throughout pregnancy



(A) Study design. Myometrial samples were taken from 4 groups of pregnant individuals: term not in labor (T-NL), term in labor (T-L), preterm not in labor (PT-NL), and preterm in labor (PT-L). **(B)** Top, agarose gel electrophoresis of the RT-PCR; bottom, relative abundance (compared to PT-NL mRNA) of mRNA encoding RHOA. **(C)** Western blot analysis of RHOA. Top, bands, and representative immunoblots revealing the expression of RHOA protein; bottom, quantification of Western Blot signals compared to PT-NL. Data are expressed as the median + standard error. N=8 in each group. PT-NL, preterm not in labor; PT-L, preterm in labor; TN-L, term not in labor; T-L, term in labor. *P<.05, **P<.01.

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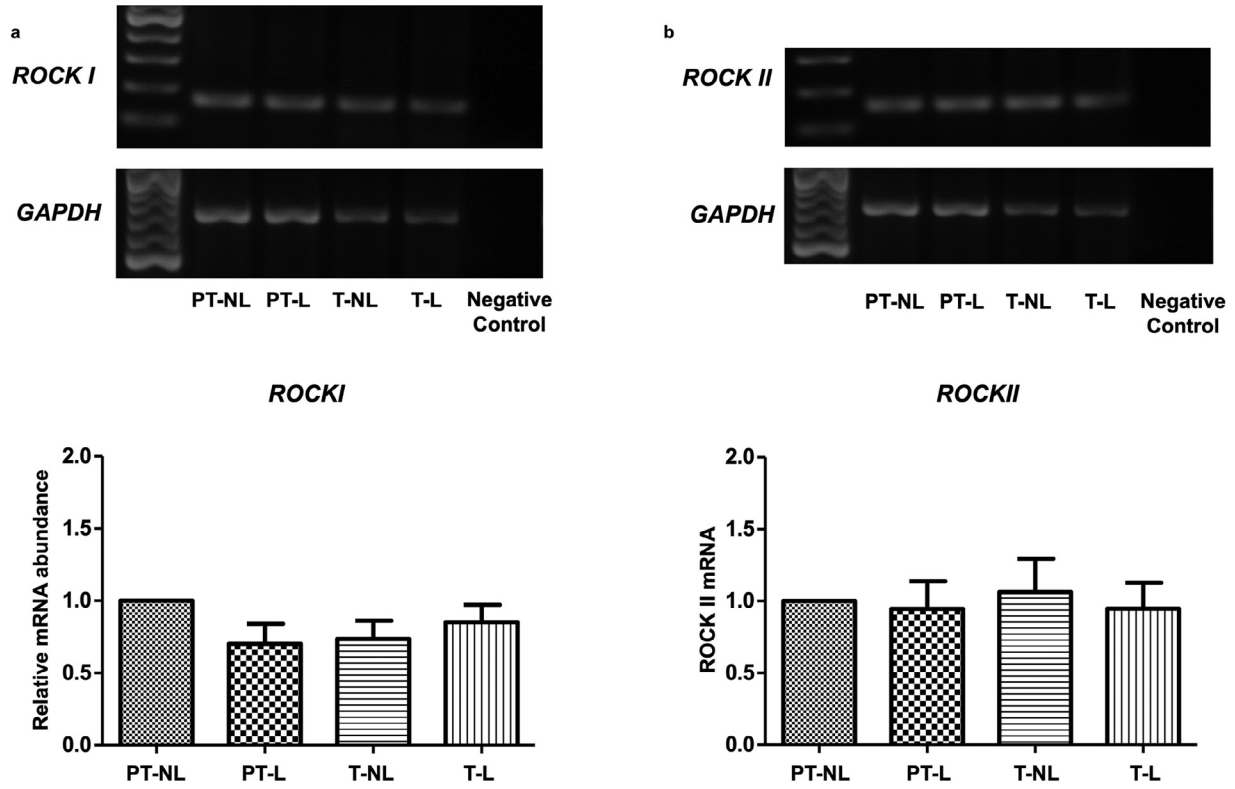
FIGURE 2
Expression of myometrial *ROCK I* and *ROCK II* throughout pregnancy



(A) Top, agarose gel electrophoresis of the RT-PCR; bottom, relative abundance (compared to PT-NL mRNA) of mRNA encoding ROCK I. **(B)** Top, agarose gel electrophoresis of the RT-PCR; bottom, relative abundance (compared to PT-NL mRNA) of mRNA encoding ROCK II. Data are expressed as the median + standard error. *N*=6 in each group. PT-NL, preterm not in labor; PT-L, preterm in labor; TN-L, term not in labor; T-L, term in labor.

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contraction.[28] Thus, to determine the activity of the ROCK pathway, western blot analysis was performed for pMYPT1. ROCK activity was estimated by measuring pMYPT1 (Thr853) compared to total MYPT1.

The levels of pMYPT1 were larger in women in labor, either at term or preterm, compared to those who delivered preterm without labor (Figure 4).

Comment

Principal findings

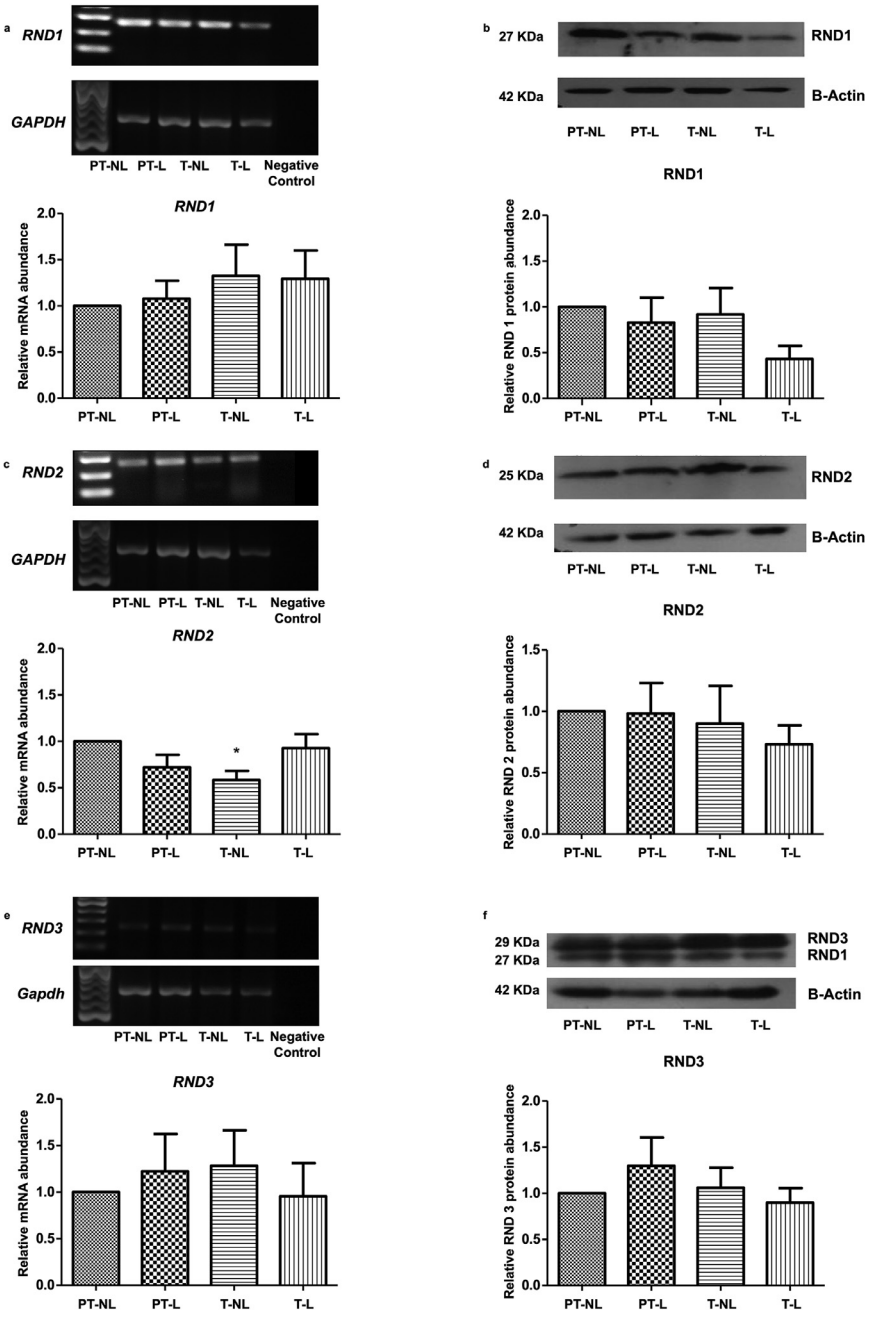
The current study showed that the RHOA/ROCK pathway components are expressed in all myometrial stages of human pregnancy. The RHOA protein and ROCK activity were increased in the myometrium from women with preterm and term labor. Besides, RND, ROCK I and ROCK II expression did not differ among the study groups. Therefore, our initial hypothesis that that the inhibition of components of the RHOA/ROCK pathway maintains uterine quiescence was not supported by our findings.

The uterine contractile activity during pregnancy and labor can be divided into four distinct physiologic stages.[2] During most of the gestation, the myometrium remains quiescent to assure fetal maturation. Myometrial quiescence precedes myometrial activation, a physiological evolution tightly regulated by several factors, including hormonal, immune, and mechanical processes, among others.[1,8,11,29–31] The myometrial stimulation stage corresponds to the clinical labor; and requires a prior myometrial activation.[2,3] Therefore, the samples we selected for this study represent myometrium from the physiological stages quiescence, activation, and stimulation, respectively preterm not in labor, term not in labor, and term in labor. Those myometrial samples obtained from women with preterm labor probably correspond to premature (i.e., nonphysiological) activation and stimulation, which may cause preterm labor.

Our findings showing the constitutive myometrial expression of the RHOA/ROCK pathway components at the mRNA and the protein level during pregnancy are consistent with previous studies.[13,14,32] However, to our knowledge, this is the first report evaluating its levels in human samples across gestation, including quiescent myometrium.

The transition from quiescence to activation depends partially on the balance of intracellular calcium and calcium sensitization.[33] For this reason, we

FIGURE 3
Expression of myometrial RHOA throughout pregnancy



(A) Top, agarose gel electrophoresis of the RT-PCR; bottom, relative abundance (compared to PT-NL mRNA) of mRNA encoding RND1. (**B)** Western blot analysis of RND1. Top, bands and representative immunoblots revealing the expression of RND1 protein; bottom, quantification of Western Blot signals compared to PT-NL. (**C)** Top, agarose gel electrophoresis of the RT-PCR; bottom, relative abundance (compared to PT-NL mRNA) of mRNA encoding RND2. (**D)** Western blot analysis of RND2. Top, bands, and representative immunoblots revealing the expression of RND2 protein; bottom, quantification of Western Blot signals compared to PT-NL. (**E)** Top, agarose gel electrophoresis of the RT-PCR; bottom, relative abundance (compared to PT-NL mRNA) of mRNA encoding RND3. (**F)** Western blot analysis of RND3. Top, bands, and representative immunoblots revealing the expression of RND3 protein; bottom, quantification of Western Blot signals compared to PT-NL. Data are expressed as the median + standard error. *N*=7 in each group. PT-NL, preterm not in labor; PT-L, preterm in labor; TN-L, term not in labor; T-L, term in labor. **P*<.05.

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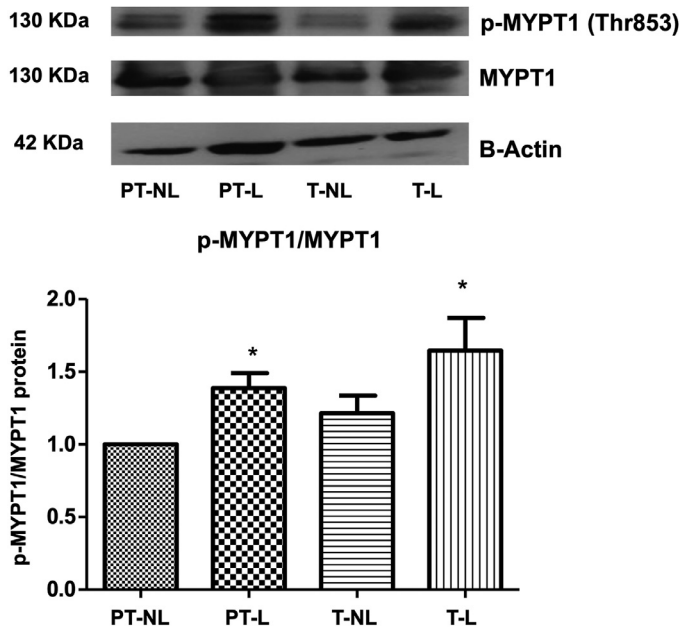
FIGURE 4
ROCK protein's activity is increased in labor



Top, bands, and representative immunoblots revealing the expression of p-MYPT1 and MYPT1 proteins; bottom, quantification of the p-MYPT1/MYPT1 ratio of Western Blot signals compared to PT-NL. Data are expressed as the median + standard error. $N=7$ in each group. PT-NL, preterm not in labor; PT-L, preterm in labor; TN-L, term not in labor; T-L, term in labor. *$P<.05$.

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studied whether the inhibition of the RHOA/ROCK pathway could be part of the mechanisms that cause myometrial quiescence. We found similar RHOA and ROCK levels in myometrial samples from quiescence and activation stages, suggesting that the RHOA/ROCK pathway's downregulation is not a part of the mechanisms underlying myometrial quiescence. A similar investigation reported that RHOA protein expression decreased in human pregnant compared to nonpregnant myometrium.[14] Therefore, the reduced RHOA protein during pregnancy would suggest a physiological role of RHOA downregulation. However, we discard that this downregulation may be related to myometrial quiescence genesis since we did not find differences between myometrial quiescence and activation. In the same study, the authors also found a higher expression of RHOA mRNA in myometrium from pregnant women in labor at term compared to term nonlabor.[14] We also showed that RHOA protein increased in women with labor at term or preterm compared to the myometrium from those who delivered preterm without labor. In line with these results, other genes that are involved in RHO activation, such as the Rho GTPase Activating Protein 4 (ARHGAP4), are part of the genes initiating term labor.[34] Notably, the myometrial RHOA protein levels are not different between preterm not in labor and term not in labor. Therefore, myometrial activation does not modify RHOA expression. We may speculate that either increased RHOA expression can trigger the onset of labor, or the onset of labor cause an increase of RHOA levels. Nevertheless, a further mechanistic investigation is required to answer this question.

In the current study, we hypothesized that myometrial quiescence could be maintained by inhibition of the RHOA/ROCK pathway by RND proteins; however, we did not find differences in the expression of RND proteins among the study groups. The RND proteins are GTP binding proteins that are members of the RHO family and lack intrinsic GTPase activity.[35] These proteins are constitutively active and can inhibit the RHOA/ROCK pathway through two mechanisms: (a) phosphorylating downstream molecules,[36] and (b) increasing the activity of RHO GTP-ase activating proteins (GAPs).[22] A seminal investigation studied the expression of RND proteins in myometrium from rats, demonstrating that RND1 inhibits Ca^{2+} sensitization by directly interfering with RHOA. This effect could be exacerbated by steroidal hormones.[21] Another study showed an increased protein expression of RND2 and RND3 in pregnant versus nonpregnant human myometrium.[24] Together, these previous studies showed the differential expression of RND proteins in pregnant and nonpregnant myometrium. However, our results demonstrated that, even though RND proteins expression is higher during pregnancy, such an expression does not change according to the different myometrial stages throughout pregnancy.

We also found that ROCK activity, measured by the phosphorylation of MYPT1, increased in the myometrium from women in labor either at term or preterm. However, the activity in quiescent and active myometrium did not differ. Therefore, pMYPT1 increased only in the stimulated myometrium. These findings are in line with a previous investigation showing that the phosphorylation of MYPT1 is ROCK-dependent and changes dynamically with each myometrial contraction in a phasic manner.[28] Moreover, the RHOA/ROCK pathway could play a role in other tissues during labor. It has been shown that there is a significant reduction of the expression of RHOA, ROCK I, and ROCK II in the cervix of pregnant mice during parturition, suggesting that the inhibition of the RHOA/ROCK pathway may be involved in cervical ripening.[37] Finally, it is well known that RHOA can serve as a critical regulator of innate and adaptive immunity;[38] therefore, as labor is an inflammatory process in nature,[29,39–41] we can speculate that the RHOA/ROCK pathway could also be involved the regulation of the immune response taking place in

the different components of the common pathway of labor.

Using functional genomics approaches, we have demonstrated that multiple molecular pathways are implicated in the regulation of uterine relaxation and contraction in humans.[42] Similarly, interactive gene networks have been identified in the guinea pig myometrium during labor,[43] suggesting that multiple therapeutics approaches could be explored. Our current results, together with previous investigations, suggest that the RHOA/ROCK pathway could serve as potential target to inhibit uterine contractions. Indeed, in vitro approaches have demonstrated divergent results in this regard. One study showed that the utilization of the RHO inhibitor Y-27632 does not reduce in vitro myometrial contractility induced by melatonin.[44] In contrast, another study demonstrated that the same inhibitor exerts a dose-dependent reduction of myometrial contraction induced by oxytocin.[13] Therefore, further research is warranted to investigate whether inhibition of the RHOA/ROCK pathway can be translated into a therapeutic tool to inhibit uterine contractions.

Research implications

Our findings, revealing a role for the RHOA/ROCK pathway in myometrial quiescence and activation, could be leveraged to further investigate clinical strategies targeting this pathway for obstetrical diseases such as preterm labor. Moreover, the inclusion of Latin individuals may serve as motivation for investigators to include this otherwise under-investigated population.

Strengths and limitations

A strength of our study is the inclusion of uterine tissue obtained from pregnant individuals at various stages of pregnancy. Moreover, all individuals were Latin, a population that is typically underrepresented in research. Another strength is the evaluation of the RHOA/ROCK pathway at both the mRNA and protein levels. However, our study is not exempted from some limitations. The number of individuals included in each study group was not substantial. Nevertheless, this limitation was due to the difficulty of obtaining myometrial samples from well-characterized pregnant individuals at different stages of gestation.

Conclusion

In conclusion, the study herein showed that the human myometrium constitutively expressed RHOA/ROCK pathway components throughout pregnancy. No changes in the components of the RHOA/ROCK pathway were found during quiescence. Moreover, the RHOA protein and ROCK activity increased in the myometrium during labor, supporting the hypothesis that this pathway participates in maintaining the contractile activity of the myometrium. This study provides insight into the role of the RHOA/ROCK pathway in controlling myometrial contractile activity during pregnancy. ■

CRediT authorship contribution statement

Jorge A. Carvajal: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Jose Galaz:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Data curation. **Sofía Villagrán:** Writing – original draft, Visualization, Investigation. **Rocío Astudillo:** Writing – review & editing, Writing – original draft, Visualization. **Liliana Garmendia:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Ana María Delpiano:** Supervision, Methodology, Investigation, Formal analysis, Data curation. ■

DATA AVAILABILITY

All data is included in the manuscript.

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