Roles and mechanisms of TRPC3 and the PLCγ/PKC/CPI-17 signaling pathway in regulating parturition

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Abstract. The aim of the current study was to investigate the role of phospholipase C (PLC)γ/protein kinase C (PKC)/C-kinase-activated protein phosphatase-1 (CPI-17) signaling pathways in uterine smooth muscle during parturition. Samples of uterine tissue were collected from pregnant patients who underwent a caesarean section for preterm delivery, full-term delivery with labor onset, full-term delivery without labor onset, and from a non-pregnant control group undergoing surgery for cervical intraepithelial neoplasia III. Immunohistochemistry, and western blotting were used to assess the association between TRPC3 levels and parturition and the influence of calcium ion channels. In addition, pregnant mice were used to explore the effect of uterine canonical transient receptor potential 3 (TRPC3) expression on the parturition-triggering mechanism and PLCγ/PKC/CPI-17 signaling pathways. Pregnant mouse uterine smooth muscle cells were cultivated, with and without TRPC3 silencing, and the expression levels of PLCγ, PKC and CPI-17, the upstream and downstream factors of the TRPC3 pathway, were measured in pregnant mouse uterine smooth muscle cells, in order to provide a theoretical basis for the prevention and treatment of premature labor. In the preterm and full-term without labor onset patient groups, the TRPC3 gene expression in the mSMCs was significantly overexpressed when compared with the non-pregnant group ($P<0.05$); however, TRPC3 expression was not elevated in the full-term with labor onset group, exhibiting no significant difference compared with the non-pregnant group ($P>0.05$). During pregnancy, compared with the non-pregnant controls, $Ca_v1.2$, $Ca_v3.1$ and $Ca_v3.2$ gene expression levels were markedly increased ($P<0.05$) in mSMCs from the preterm delivery group and the full-term with labor onset group, however were non-significantly increased in the full-term without labor onset group. The level of TRPC3 was highest in the preterm group, while the levels of $Ca_v1.2$, $Ca_v3.1$ and $Ca_v3.2$ were highest in the full-term with labor onset group. In the preterm, LPS-treated preterm and full-term groups, TRPC3, MAPK, ERK1/2, P-ERK, $Ca_v3.2$, $Ca_v3.1$ and $Ca_v1.2$ were all expressed at higher levels than in the unfertilized group. In the LPS-treated preterm group, the levels of TRPC3, MAPK, ERK1/2, P-ERK, $Ca_v3.2$, $Ca_v3.1$ and $Ca_v1.2$ were increased compared with the preterm group. Furthermore, following transfection of small interfering TRPC3 (siTRPC3) into cells, it was demonstrated that the levels of TRPC3, PLCγ, PKC, CPI-17, P-CPI-17, $Ca_v1.2$, $Ca_v3.1$ and $Ca_v3.2$ expression were lower in the LPS siTRPC3 group when compared with that of the LPS-treated untransfected control group.

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Introduction

The mechanism that triggers childbirth is complex and remains to be fully elucidated. Previous studies have demonstrated that parturition involves interactions between various processes, factors, regulatory pathways and molecules, which comprise multi-stage changes (1,2). Various theories regarding the mechanisms of the triggering of childbirth, including neurotransmission, mechanical changes, endocrine control, cervical maturity and lower uterine segment formation, in addition to immunological theories, have been proposed. It is widely accepted that normal childbirth relies on the rhythmic contraction of the uterine smooth muscle and progressive expansion of the cervix. Additionally, various tissues, organs and factors are involved in the process of parturition; this includes endocrine and paracrine signaling by the mother and the fetus, involving hormones secreted from the placenta, fetal membranes, uterus, decidua and other adjacent tissues, cytokines produced in the uterus, and smooth muscle cell membrane receptors. Alterations in these signaling mechanisms ultimately induce contraction of the uterine smooth muscle, leading to parturition (3-6). This evidence suggests that uterine smooth muscle contraction is a crucial trigger of the childbirth process.

An intracellular calcium signaling mechanism has been proposed that induces periodic uterine contractions; this mechanism involved binding between hormone and receptor, receptor and G protein activation, and activation of adenylyl cyclase and phospholipase C (PLC). Adenylyl cyclase

catalyzes the production of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP), resulting in the activation of cAMP-dependent protein kinases. PLC on the membrane surface catalyzes the formation of diacylglycerol (DAG) and inositol triphosphate (IP_3) from phosphatidylinositol-4,5-bisphosphate (PIP_2). DAG activates protein kinase C (PKC), and PKC subsequently activates voltage-dependent calcium channels, leading to extracellular calcium influx. IP_3 binds with the IP_3 receptor on the endoplasmic reticulum (ER) surface, which induces the release of calcium from the ER to increase cytoplasmic calcium levels. Calcium binds with calmodulin, which causes activation of myosin light chain (MLC) kinase, and phosphorylation of the head of the MLC filament; this facilitates the contraction of smooth muscle. Increasing intracellular calcium often depends on the transmembrane transfer of extracellular calcium into cells, which can trigger further release of calcium from intracellular stores. Ca^{2+} uptake and release by calcium channels in the plasma membrane and intracellular sarcoplasmic reticulum (SR) mediate intracellular calcium homeostasis. Thus, calcium channels serve a crucial role in the regulation of smooth muscle activity. Under normal circumstances, extracellular Ca^{2+} predominantly enters cells through calcium channels in the cell membrane, which triggers further Ca^{2+} release from the SR, leading to uterine contractions.

Classical transient receptor potential (TRP) channels, as six-transmembrane non-selective cation channels, participate in numerous physiological and pathological processes, including cell proliferation, angiogenesis, neuronal morphogenesis and synaptogenesis, and tumor formation. Sodium, calcium and magnesium ions can enter cells through canonical TRP (TRPC) channels, which respond to diverse external stimuli. Human cells express six TRPC isoforms: TRPC1, 3, 4, 5, 6 and 7 (TRPC2 is a pseudogene in humans). The TRPC family can be classified into four subgroups according to structural homology and functional predisposition as follows: TRPC1; TRPC2; TRPC4 and 5; and TRPC3, 6 and 7 (7-10). TRPC channels are expressed during embryonic development and adulthood. Previous studies have detected the expression of TRPC channels in the mammalian myometrium, and mechanical contraction of human uterine smooth muscle has been demonstrated to cause upregulation of TRPC3 expression.

There are two mechanisms that activate TRP channels resulting in calcium influx: Store-operated calcium entry (SOCE) and receptor-operated calcium entry (ROCE). TRP channels can be activated by G protein-coupled receptors and tyrosine kinase receptors, or PLCβ and PLCγ. Following activation of a phosphatidylinositol-specific or PLC-specific G protein-coupled receptor, or a PLCγ-specific receptor tyrosine kinase, PLC is induced to hydrolyze PIP_2 into IP_3 and DAG (11,12). IP_3 acts on the ER or SR, leading to the release of Ca^{2+}. In SOCE (formally known as capacitative calcium entry), when calcium stores are depleted, TRPC channels on the cell membrane open to allow an influx of extracellular Ca^{2+}. By contrast, ROCE is mediated by DAG, which directly activates TRPC channel opening.

PKC is a family of Ca^{2+}-activated phospholipid-dependent serine/threonine protein kinases that are widely distributed in various organs. PKCs regulate processes in various cell types, including gene expression, cell proliferation, apoptosis and cell migration (13). The PKC/PKC-potentiated phosphatase inhibitor protein of 17 kDa (CPI-17) signaling pathway serves a critical role in the calcium sensitization of smooth muscle. CPI-17, a key substrate of PKC, is a PKC-dependent phosphatase inhibitor that inhibits the enzymatic activity of myosin light chain phosphatase (MLCP) (14,15). Su *et al* (16) silenced CPI-17 gene expression in bronchial smooth muscle using RNA interference technology, which decreased bronchial smooth muscle calcium sensitization, contraction frequency and contractility, suggesting that CPI-17 affects the contraction of smooth muscle. Jiang *et al* (17) reported that PKC agonists significantly increased the phosphorylation of myosin light chain (MLC) and the contraction of vascular smooth muscle. Due to specific MLCP inhibition, exogenous phosphorylated CPI-17 increases vascular smooth muscle contraction in a dose-dependent manner. These results suggest that PKC activation inhibits MLCP activity by inducing CPI-17 phosphorylation, thereby increasing MLC20 phosphorylation and the calcium sensitivity of contractile proteins, leading to vascular and bronchial smooth muscle contraction (13,18).

TRPC channels can mediate extracellular calcium influx and induce uterine contraction, and additionally exert extensive physiological effects in the nervous system, vascular smooth muscle, myocardium and skin. L-type and T-type calcium channels can enhance the function of TRPC3 channels and can promote the contraction of uterine spiral arteries in pregnant mice during parturition. PLCγ can activate TRPC channels and PKC, and the PKC/CPI-17 pathway serves an important role in vascular smooth muscle contraction and calcium sensitization. Therefore, it was hypothesized that TRPC3 triggers parturition via L-type and/or T-type calcium channels, and that the PLCγ/PKC/CPI-17 pathway is the major regulator involved in inducing parturition.

In the present study, uterine samples were collected from patients that experienced preterm delivery with or without labor onset, full-term delivery with labor onset, full-term delivery without labor onset, and from a non-pregnant control group. The effects of TRPC3 on labor onset and on calcium channel expression were detected by immunohistochemistry and western blotting. Additionally, pregnant mice were treated with either a TRPC3 inhibitor or agonist, and uterine samples were harvested at different time-points in an attempt to investigate the underlying role of TRPC3 in parturition and its influence on PLCγ/PKC/CPI-17 signaling. Uterine smooth muscle cells from pregnant mice were isolated, cultured and treated with TRPC3 inhibitors, then the expression of factors upstream and downstream of TRPC3 were detected to determine the mechanisms of PLCγ/PKC/CPI-17 in the regulation of TRPC3 functions in uterine smooth muscle cells from pregnant mice. The results may provide evidence that could be useful for the prevention and treatment of premature childbirth.

Materials and methods

Ethics statement. The present study was approved by the Clinical Research Ethics Committee of Shenyang Hospital of China Medical University (Shenyang, China). Written informed consent was obtained from all patients.

Tissue and serum collection. A total of 80 tissue samples (4 groups, 20 samples per group: Preterm delivery with or without labor onset, full-term delivery with labor onset, full-term delivery without labor onset and non-pregnant controls) were obtained from patients who underwent surgery [caesarean section (pregnant groups) or treatment for cervical intraepithelial neoplasia III (non-pregnant control group)] at Shenyang Hospital of China Medical University between October 2015 and June 2016. Full-term delivery was defined as parturition at 38-40 weeks of gestation, and labor onset was defined as the presence of regular contractions and/or cervical dilation of 2-3 cm. All collected tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80˚C. After placentas were collected from the pregnant women, whole blood samples were taken from the placenta and subjected to serum separation by centrifugation, then stored at a low temperature.

Mouse strains. A total of 80 adult female Kunming mice were subjected to timed matings. The morning that vaginal plugs were detected was designated as day 0 of pregnancy. Mice were housed in a 25˚C temperature room with alternating 12-hour light and dark cycles and had free access to food and water.

Murine preterm labor model. Mice were randomly divided into an unfertilized group (group A), an 'infected' [lipopolysaccharides (LPS)-treated] preterm group (group B), a 15-day gestation (preterm cesarean section group (group C), and a full-term cesarean section group (group D).

At day 15, the pregnant mice in group B received four injections of LPS in salt solution (350 μg/kg) into the celiac artery, at intervals of 3 h. In all the groups, mice were injected with a TRPC3 channel inhibitor SKF96365 (ab120280; Abcam, Cambridge, UK) or saline (1 h after the injection of group B with LPS) and the litter delivery was observed and recorded. The mice were closely observed 24 h/day to record premature deliveries (i.e., parturition earlier than at day 19). When parturition occurred in the pregnant mice (full delivery of first offspring), the uterine tissue was immediately obtained; 10% chloral hydrate (dose 0.03 ml/kg) was used for anesthetization and the mice were then sacrificed by cervical spinal dislocation. The obtained uterine and adipose tissues were irrigated with saline solution and stored at -80˚C.

Plasmid and small interfering RNA (siRNA) transfection. A TRPC3 construct was generated by subcloning the TRPC3 coding sequence into a pcDNA3.0 vector using standard subcloning protocols. In addition, synthetic siRNAs of 17- or 19-nucleotide duplex RNA and 2-nucleotide 3'-dTdT overhangs were designed and obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The green fluorescence-tagged sequences of siRNAs used in the present study were as follows: Control, 5'-UUCUCCGAACGUGUCACG U-3'; mouse TRPC3-1, 5'-AGCCUUCUGUGCUGAGAAC-3'; and mouse TRPC3-2, 5'-GGACCGCAAAGUGUUUGUG-3'. In order to silence the expression of TRPC3, cells at 60-70% confluence were seeded overnight and transfected with either 5 μl of 20 μM siRNA (final concentration 50 nM) against TRPC3 or control siRNA (non-target scramble siRNA) with Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in OptiMEM (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Transfection was confirmed as following 3 days, green fluorescence was observable under the fluorescence microscope. Cells were used in subsequent assays 48 h following transfection.

Confocal microscopy. Uterine smooth muscle cells were fixed in confocal chambers using 6% formaldehyde for 15 min at room temperature, followed by permeabilization with 0.1-0.2% Triton X-100 for 20 min. After washing, the cells were incubated with the appropriate primary antibody [TRPC3 (rabbit, 25 kDa; 1:1,000; cat. no. ab51560; Abcam, Cambridge, UK), PLCγ (rabbit, 1:1,000; Sigma-Aldrich; Merck KGaA; cat. no. 05-163), PKC (rabbit, 1:1,000; Abcam; cat. no. ab31), CPI-17 (rabbit, 17 kDa; 1:1,000; Abcam; cat. no. ab131451), P-CPI-17 (rabbit, 17 kDa, 1:1,000; Abcam; cat. no. ab52174), Cav1.2 (rabbit, 239 kDa, 1:1,000;Abcam;cat.no.ab58552),Cav3.1(rabbit,262kDa,1:1,000; Abcam; cat. no. ab203577), Cav3.2 (rabbit, 262 kDa; 1:1,000; Abcam; cat. no. ab128251)] at 4˚C overnight followed by incubation at room temperature for 1 h with a secondary antibody (1:1,000; Abcam; cat. no. ab6721). Confocal images were obtained using a Zeiss LSM510 laser scanning microscope (Zeiss, Oberkochen, Germany) with single (488 nm) or multitrack sequential excitation (488 and 633 nm), and emission [515-540 nm, boron dipyrromethene fluorescein; 650 nm, Alexa Fluor 633] filter sets (Chroma Technology Corporation, Bellow Falls, VT, USA). The color of 488 nm excitation was set as pseudo green, and the color for 633 nm excitation line was set as pseudo red. The merge of these two colors (pseudo green and pseudo red) yielded yellow when colocalization of two proteins occurred.

Western blotting. Samples from the four patient groups were ground using a pestle and mortar and then resuspended in PBS (pH 7.4) containing a protease inhibitor cocktail (Roche Diagnostics, Castle Hill, Australia). The proteins were centrifuged (3,000 x g, 4˚C, 5 min) and the supernatant was separated from the pellet then placed on ice. The pellet was snap frozen and reprocessed as stated above, and supernatants from each group were pooled and centrifuged (20,000 x g, 4˚C, 1 h). Protein extracts were aliquoted, rapidly frozen in liquid nitrogen, and stored at -80˚C. A Bradford protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to determine the protein concentration in samples.

Protein extracts were dissolved in lysis buffer with a protease inhibitor cocktail, and then separated by 10% SDS-PAGE and electroblotted onto PVDF membranes for 2 h (80 V) at 4˚C. PVDF membranes were then washed with PBS, blocked and probed with anti-TRPC3 (rabbit, 25 kDa; cat. no. ab51560; 1:1,000; Abcam) anti-p38 MAPK antibody (cat. no. ab197348; 1:1,000; Abcam) and anti-ERK 1/2 (phospho-Thr202/Tyr204; cat. no. ab214362; 1:1,000; Abcam) antibody overnight at 4˚C. Specific binding was visualized using alkaline phosphate-conjugated secondary antibodies (Abgent; 1:1,000; cat. no. ARS3337) and chemiluminescence, according to manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). MagicMark XP Western Protein Standard (Invitrogen; Thermo Fisher

Scientific, Inc.) was used to estimate the migrated protein band sizes. The intensity of bands corresponding to protein expression was determined using Photoshop CS3 Extended software (Adobe Systems, Inc., San Jose, CA, USA). Relative levels of protein expression were determined by normalizing the intensity of TRPC3 staining on the blots to avoid potential changes in SMC protein expression in pregnancy, with a GAPDH primary antibody (mouse, 37 kDa, 1:5,000; cat. no. sc-25778; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) used as the loading control. Notably, extensive attempts to undertake western blot protocols using antibodies against L-type and T-type calcium channel subunits $Ca_v1.2$ (rabbit, 239 kDa, 1:1,000; Abcam; cat. no. ab58552), $Ca_v3.1$ (rabbit; 262 kDa; 1:1,000; Abcam; cat.no.ab203577), $Ca_v3.2$ (rabbit, 262 kDa; 1:1,000; Abcam; cat. no. ab128251) antibodies to detect proteins at the respective molecular weights.

Cell isolation and primary culture of mouse myometrial smooth muscle cells (mSMCs). Strips of longitudinal muscle (10-20 mm long) were cut from the serosal surface of the uterine horn of mouse uteri, carefully excluding the endometrium and circular muscle, and finely cut into 2-3 mm-long pieces in ice-cold HBSS and then incubated with gentle shaking for 40 min at 37˚C in 5 ml HBSS containing 1.5 mM $CaCl_2$, collagenase type IA (2 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), trypsin inhibitor (2 mg/ml; soybean, type II-S; Sigma-Aldrich; Merck KGaA), and bovine serum albumin (6 mg/ml; Sigma-Aldrich; Merck KGaA). The cell suspension was then triturated 15 to 20 times, filtered through a 40-mm sterile filter, and washed twice in minimum essential medium (MEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal calf serum (FCS; Gibco; Thermo Fisher Scientific, Inc.) with centrifugation (450 x g, 5 min, 20˚C). The cell pellet was suspended in MEM supplemented with 5% FCS, penicillin (25 U/ml) and streptomycin (25 U/ml); (Gibco; Thermo Fisher Scientific, Inc.). For imaging protocols, a cell solution (400 ml) was placed on glass coverslips and cells were allowed to adhere for ~1 h, after which the coverslips were flooded with an additional 1.6 ml of culture medium. For western blot analysis, myocytes were seeded into 25-cm^3 culture flasks. Myocytes were incubated at 37˚C in a humidified atmosphere of 95% air/5% CO_2 and maintained as a primary culture until confluent (4 days on average). The culture medium was changed every 2-3 days. To assess the purity of myocyte cultures, immunocytochemistry was performed using α-actin (Sigma-Aldrich; Merck KGaA) and calponin (Abcam) monoclonal antibodies.

Uterine smooth muscle tissue samples from the preterm delivery, full-term delivery with labor onset, full-term delivery without labor onset, and non-pregnant control groups were placed in sterile petri dishes, and rinsed 2-3 times with saline solution. Fibrous connective tissue was then removed: The samples were cut into small pieces and placed in a small 50-ml beaker, then subjected to trypsin and collagenase I digestion to purify the fibroblasts and smooth muscle cells according to differences in their adherence times; purification was performed 2-3 times.

Immunohistochemistry. The distributions of TRPC3, $Ca_v1.2$, $Ca_v3.1$, and $Ca_v3.2$ were examined in the smooth muscle tissues from the four groups of human uterine samples using conventional whole-mount immunohistochemistry and confocal microscopy. Fixed whole-mount tissues were incubated in blocking buffer (PBS containing 1% bovine serum albumin and 0.2% Tween-20; Sigma-Aldrich; Merck KGaA) for 2 h at room temperature, rinsed (3-5 min) in PBS, and incubated with primary antibodies against TRPC3, $Ca_v1.2$, $Ca_v3.1$ or $Ca_v3.2$ (1:100; Abcam) in blocking buffer for 18 h at 4˚C. Tissue was then rinsed (3-5 min) in PBS and incubated in secondary antibody (Alexa Fluor 633; Invitrogen; Thermo Fisher Scientific, Inc.) diluted in PBS containing 0.01% Tween-20 for 2 h, then rinsed again (3-5 min) in PBS, and mounted in buffered antifade glycerol (AppliChem, GmbH, Darmstadt, Germany). Tissues were examined with a confocal microscope (FV1000; Olympus Corporation, Tokyo, Japan) and images were acquired with uniform settings.

Immunofluorescent staining of myometrial tissue. Uterine tissue was obtained from pregnant and unfertilized mice, placed in optimal cutting temperature (Beijing Solarbio Science & Technology Co., Ltd., Beijing) medium and frozen. Frozen tissue sections were rehydrated and treated for 30 min with 1X PBS/0.25% Triton X-100 solution, and subsequently rinsed in PBS twice for 5 min. Tissue sections were then incubated for 40 min in undiluted Sea Block Blocking Solution (Thermo Fisher Scientific, Inc.), rinsed in PBS and incubated in undiluted Fc Receptor Blocker solution (Innovex Biosciences, Inc., Richmond, CA, USA) for 30 min. After rinsing in PBS, tissue sections were incubated overnight at 4˚C with a primary antibody against TRPC3 (1:300; no. ab51560; Abcam). Sections were then washed in PBS three times for 10 min and then incubated with the suitable fluorochrome-labeled secondary antibodies (goat anti-rabbit Alexa Fluor 488; cat. no. ab150077; Invitrogen/Molecular Probes; Thermo Fisher Scientific, Inc.). Chromatin was counterstained with Hoechst (10 mg/ml; Sigma-Aldrich; Merck KGaA) diluted in H_2O (1:10,000). Specimens were examined by confocal microscopy with an Olympus FV-1000 laser scanning confocal microscope (HeNe/Ar) with a 60x water immersion objective, and images were processed with the FluoView software (Olympus Corporation). Selected representative fields are presented.

Statistical analysis. Data are expressed as the means ± standard error, with n defined in the figure legend or text as the number of animals used to obtain the data; however, as detailed in the figure legends, tissues were pooled for western blot analysis. Statistical significance was determined by one-way analysis of variance followed by Dunnett's post hoc test for multiple comparisons. Comparison of two variables was performed with unpaired Student's t-tests where appropriate, with $P<0.05$ considered to indicate a statistically significant difference (GraphPad Prism; GraphPad Software, Inc., La Jolla, CA, USA).

Results

Expression levels of TRPC3, $Ca_v1.2$, $Ca_v3.1$ and $Ca_v3.2$ increase during pregnancy. TRPC3 can be activated by stretch, swelling, heat or pressure, which are physiological stimuli that are likely to be involved in uterine contractility. Thus,

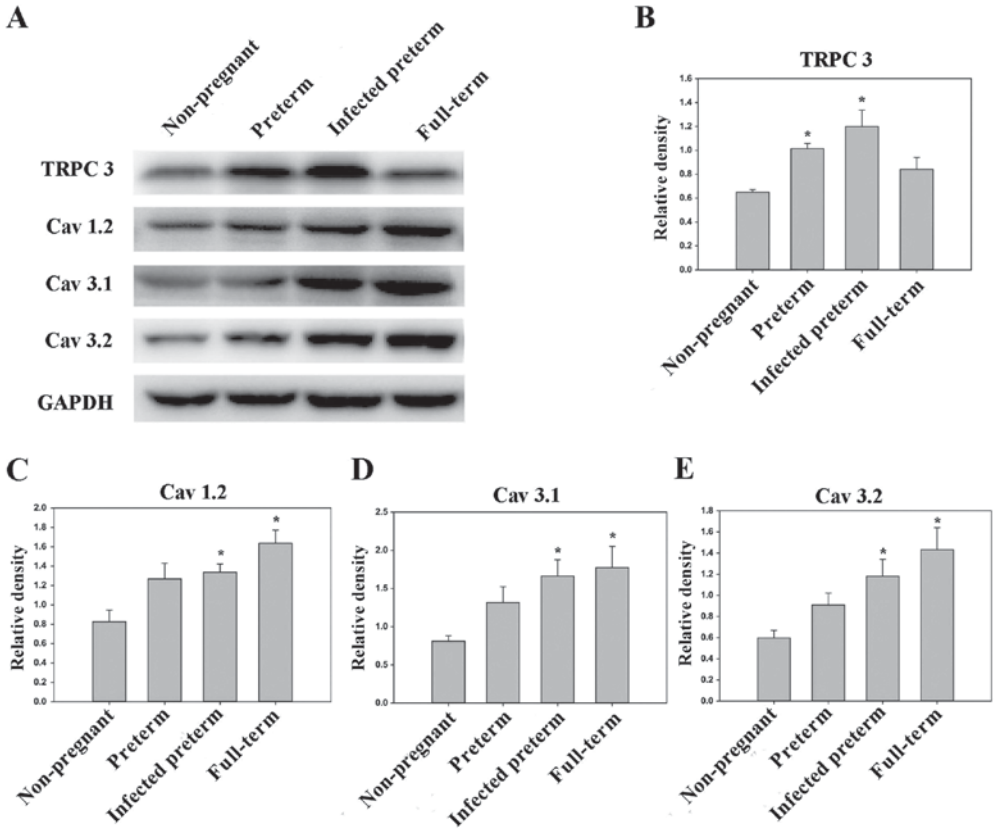

Figure 1. Expression levels of TRPC3, $Ca_v1.2$, $Ca_v.3.1$ and $Ca_v3.2$ in human myometrial smooth muscle cells derived from the non-pregnant, full-term without labor onset, preterm, and full-term with labor onset patient groups (n=20/group). (A) Western blot analysis of protein expression levels in the different groups. GAPDH was used as the loading control. Quantified western blot analyses of relative expression levels of (B) TRPC3, (C) $Ca_v1.2$, (D) $Ca_v.3.1$ and (E) $Ca_v3.2$. Data are presented as the mean ± standard error. *P<0.05 vs. non-pregnant group. TRPC3, canonical transient receptor potential 3.

to determine whether TRPC3 channels modulate myometrial tone during pregnancy, western blotting was used to measure TRPC3 gene expression in primary mSMCs obtained from human samples (preterm delivery, full-term delivery without labor onset, full-term delivery with labor onset, and non-pregnant control groups; Fig. 1). Western blotting indicated that TRPC3 protein expression was markedly higher in mSMCs from the full-term without labor onset group and preterm group compared with samples from the non-pregnant group (Fig. 1B; P<0.05); however TRPC3 expression was not significantly different in the full-term with labor onset group compared with the non-pregnant control group (P>0.05). During pregnancy, compared with the non-pregnant controls, $Ca_v1.2$, $Ca_v3.1$ and $Ca_v3.2$ protein expression levels were markedly increased (P<0.05) in mSMCs from the preterm delivery group and the full-term with labor onset group (Fig. 1C-E), however were non-significantly increased in the full-term without labor onset group. The level of TRPC3 expression was highest in the preterm group, whereas the levels of $Ca_v1.2$, $Ca_v3.1$ and $Ca_v3.2$ were highest in the full-term with labor onset group, and western blotting results were consistent with each other (Fig. 1).

Proteins were subsequently extracted from mouse uteri and analyzed by western blotting. The levels of TRPC3, mitogen-activated protein kinase (MAPK), phosphorylated (P-) extracellular signal-related kinase (ERK), $Ca_v3.2$, $Ca_v3.1$, $Ca_v1.2$ were all significantly higher in the LPS-treated preterm group and full-term group compared with the unfertilized group (all P<0.05). Additionally, the levels of MAPK and P-ERK were significantly higher in the preterm group than in the unfertilized group (P<0.05). Furthermore, the levels of TRPC3, MAPK, P-ERK, $Ca_v3.2$, $Ca_v3.1$, $Ca_v1.2$ were all higher in the LPS-treated preterm group than in the preterm group, to varying extents (Fig. 2).

Mouse uterine SMCs were transfected with the TRPC3 plasmid. After 3 days, green fluorescence was observable under the fluorescence microscope, and cell growth was normal (Fig. 3). Additionally, mouse uterine SMCs were transfected with TRPC3-1 and TRPC3-2 siRNA for 3 days, and western blot analysis was subsequently used to detect TRPC3 expression. It was observed that TRPC3 expression was decreased by transfection with either of the TRPC3 siRNA compared with the control siRNA (Fig. 3).

Effect of siRNA-mediated TRPC3 downregulation. To assess the role of TRPC3 in mSMCs, TRPC3 was downregulated by siRNA silencing. Transfection of the mSMCs with anti-TRPC3 siRNA effectively depleted TRPC3 protein (Fig. 3). Immunofluorescence analysis indicated that knockdown of TRPC3 expression reduced the expression levels of PLCγ, PKC, CPI-17, P-CPI-17, $Ca_v1.2$, $Ca_v3.1$ and $Ca_v3.2$ in mSMCs (Figs. 4-11). In mSMCs obtained from the LPS-treated group, higher expression levels of TRPC3, PLCγ, PKC, CPI-17, P-CPI-17, $Ca_v1.2$, $Ca_v3.1$ and $Ca_v3.2$ were detected in the control siRNA-transfected group compared with the TRPC3-siRNA-transfected group (Figs. 4-11). Additionally,

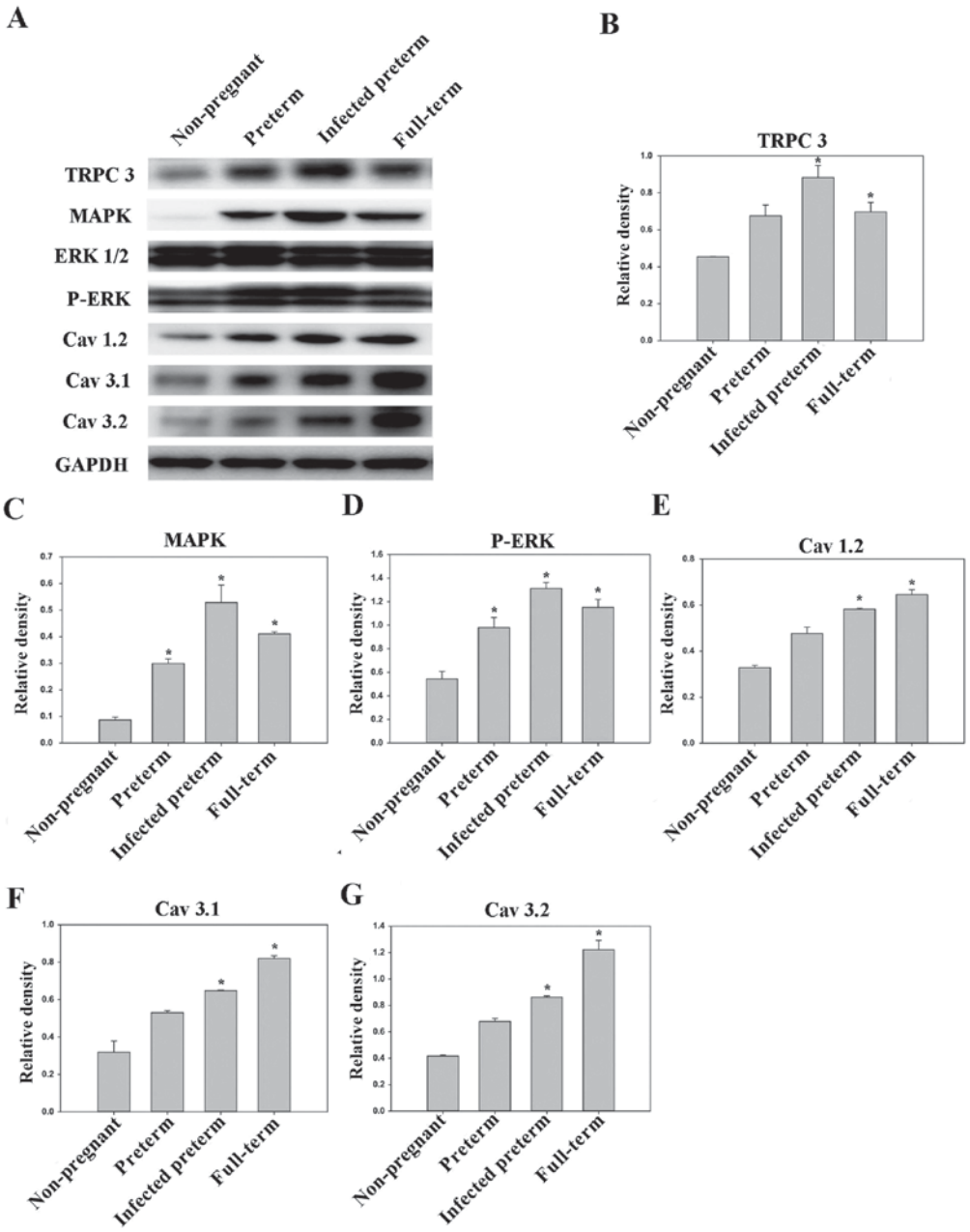

Figure 2. Expression levels of TRPC3, MAPK, P-ERK, $Ca_v1.2$, $Ca_v3.1$ and $Ca_v3.2$ in mouse myometrial smooth muscle cells derived from the non-pregnant, preterm, infected preterm and full-term groups (n=10/group). (A) Western blot analysis of protein expression levels in the different groups. GAPDH was used as the loading control. Quantified protein expression levels of (B) TRPC3, (C) MAPK, (D) P-ERK, (E) $Ca_v1.2$, (F) $Ca_v3.1$ and (G) $Ca_v3.2$. Data are presented as the mean ± standard error. *P<0.05 vs. non-pregnant group. TRPC3, canonical transient receptor potential 3; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-related kinase; P-, phosphorylated.

quantified western blot analysis confirmed that TRPC3 knock-down reduced the expression levels of TRPC3, PLCγ, PKC, CPI-17, P-CPI-17, $Ca_v1.2$, $Ca_v3.1$ and $Ca_v3.2$ compared with the control siRNA-transfected group in non-LPS-treated cells (Fig. 12).

Discussion

The effect of uterine canonical TRPC3 expression affected the parturition-triggering mechanism and PLCγ/PKC/CPI-17 signaling pathways. In the preterm and full-term without labor onset patient groups, TRPC3 gene expression in the mSMCs was overexpressed compared with the non-pregnant group; however, TRPC3 expression was not elevated in the full-term with labor onset group, exhibiting no difference compared with the non-pregnant group. During pregnancy, compared with the non-pregnant controls, $Ca_v1.2$, $Ca_v3.1$ and $Ca_v3.2$ gene expression levels were markedly increased in mSMCs in the preterm delivery group and the full-term with labor onset group, however were non-significantly increased in the full-term without labor onset group. The level of TRPC3 was highest in the preterm group, while the levels of $Ca_v1.2$, $Ca_v3.1$ and $Ca_v3.2$ were highest in the full-term with labor onset group. LPS-treated preterm and full-term groups, TRPC3, MAPK, ERK1/2, P-ERK, $Ca_v3.2$, $Ca_v3.1$ and $Ca_v1.2$ were all expressed at higher levels than in the unfertilized group. In

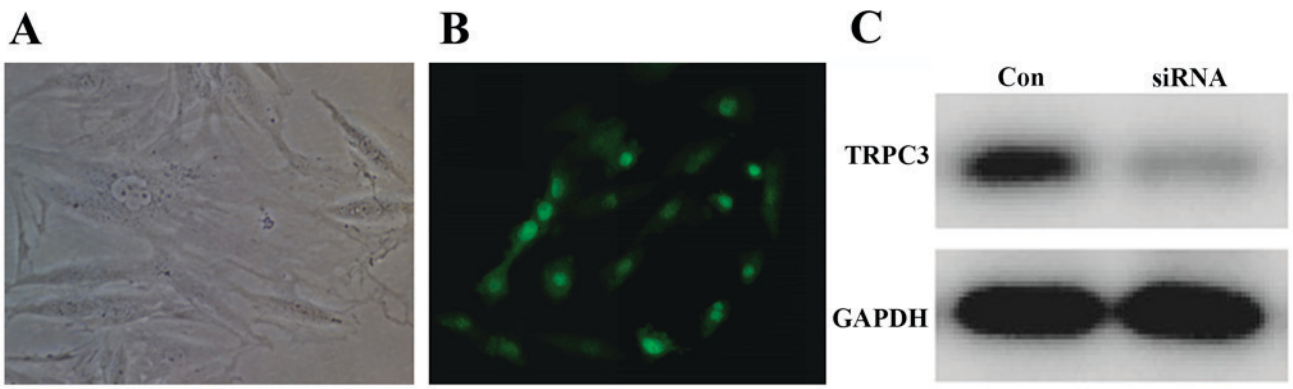

Figure 3. Immunostaining of smooth muscle actin in mouse uterine smooth muscle cells, and transfection with TRPC3 siRNA. (A) Mouse uterine smooth muscle cells. (B) Immunostaining of TRPC3 in mouse uterine smooth muscle cells. (C) The expression of TRPC3 detected by western blotting. TRPC3, canonical transient receptor potential 3; siRNA, small interfering RNA.

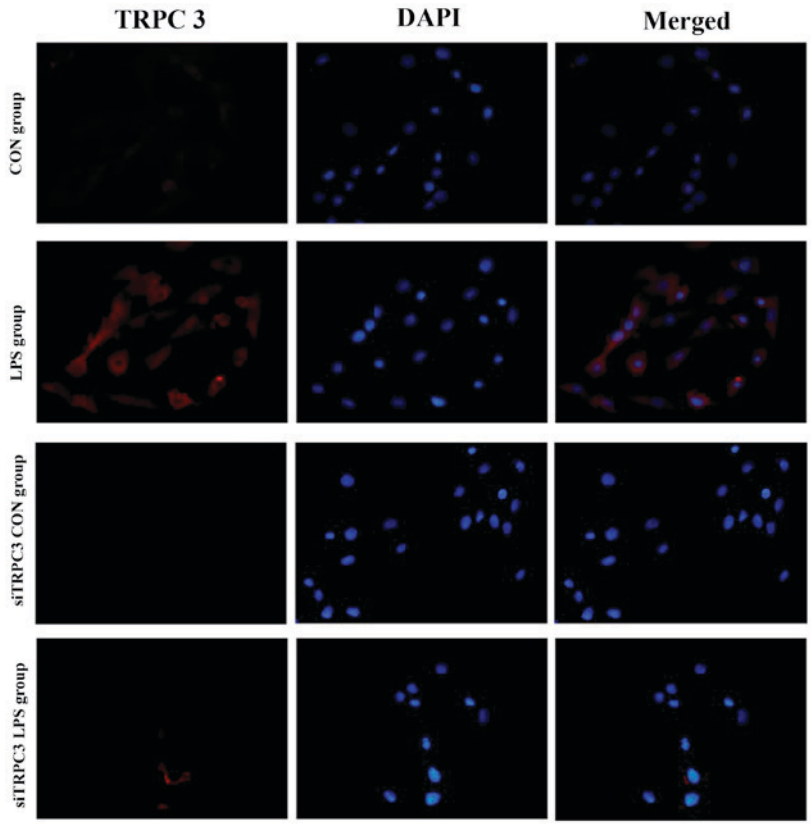

Figure 4. Expression of TRPC3 protein with and without TRPC3-siRNA transfection of murine uterine smooth muscle cells. CON, untransfected non-infected with LPS; LPS, infected with LPS; siTRPC3 CON, transfected with siTRPC3 and non-infected with LPS; siTRPC3 LPS, transfected with siTRPC3 and infected with LPS. TRPC3, canonical transient receptor potential 3; siRNA, small interfering RNA; LPS, lipopolysaccharides; CON, control.

the LPS-treated preterm group, the levels of TRPC3, MAPK, ERK1/2, P-ERK, $Ca_v3.2$, $Ca_v3.1$ and $Ca_v1.2$ were increased compared with the preterm group. Furthermore, following transfection of siTRPC3 into cells, it was demonstrated that the levels of TRPC3, PLCγ, PKC, CPI-17, P-CPI-17, $Ca_v1.2$, $Ca_v3.1$ and $Ca_v3.2$ expression were decreased in the LPS siTRPC3 group compared with the LPS-treated untransfected control group.

CPI-17 is an important signaling molecule involved in the regulation of calcium sensitivity in smooth muscle contraction. It is a PKC-activated phosphatase inhibitor with a relative molecular weight of 17 kDa, and is widely expressed in vascular and visceral smooth muscle cells (11). Studies have demonstrated that PKC phosphorylates CPI-17 at Thr-38, then phosphorylated CPI-17 binds with the 38 kDa MLCP catalytic subunit (the main unit responsible for enzymatic activity), which causes a 1,000-fold inhibition of MLCP, thus increasing calcium sensitivity and smooth muscle contraction (12-14). Additionally, nitric oxide (NO) binds with soluble guanylate cyclase in smooth muscle cells, which increases cyclic guanosine monophosphate (cGMP) levels; this activates a cGMP-dependent kinase, which then dephosphorylates CPI-17

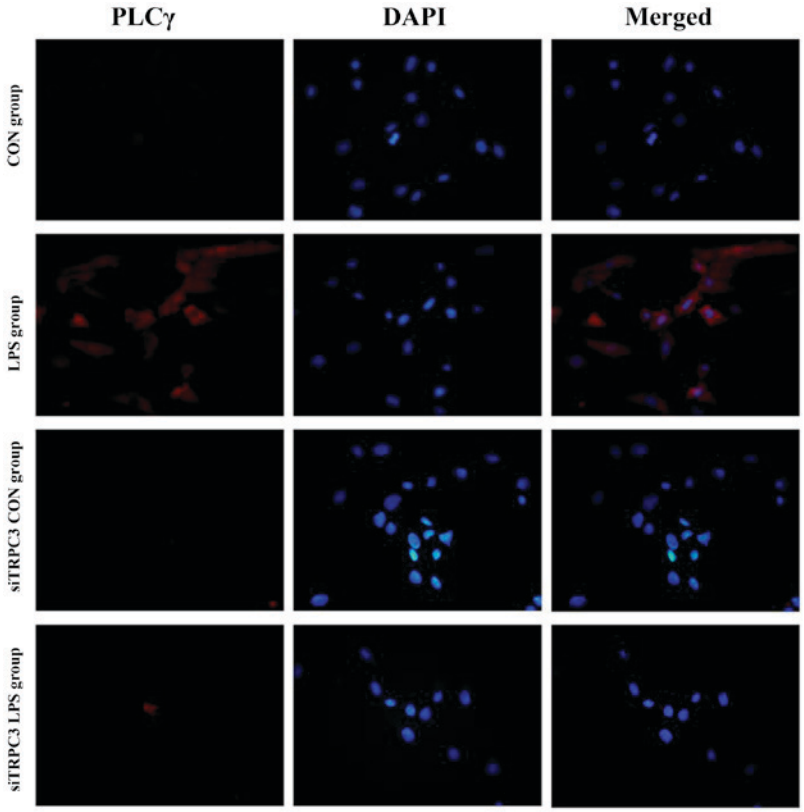

Figure 5. Expression of PLCγ protein with and without TRPC3-siRNA transfection in murine uterine smooth muscle cells. CON, untransfected non-infected with LPS; LPS, infected with LPS; siTRPC3 CON, transfected with siTRPC3 and non-infected with LPS; siTRPC3 LPS, transfected with siTRPC3 and infected with LPS. PLC, phospholipase C; TRPC3, canonical transient receptor potential 3; siRNA, small interfering RNA; LPS, lipopolysaccharides; CON, control.

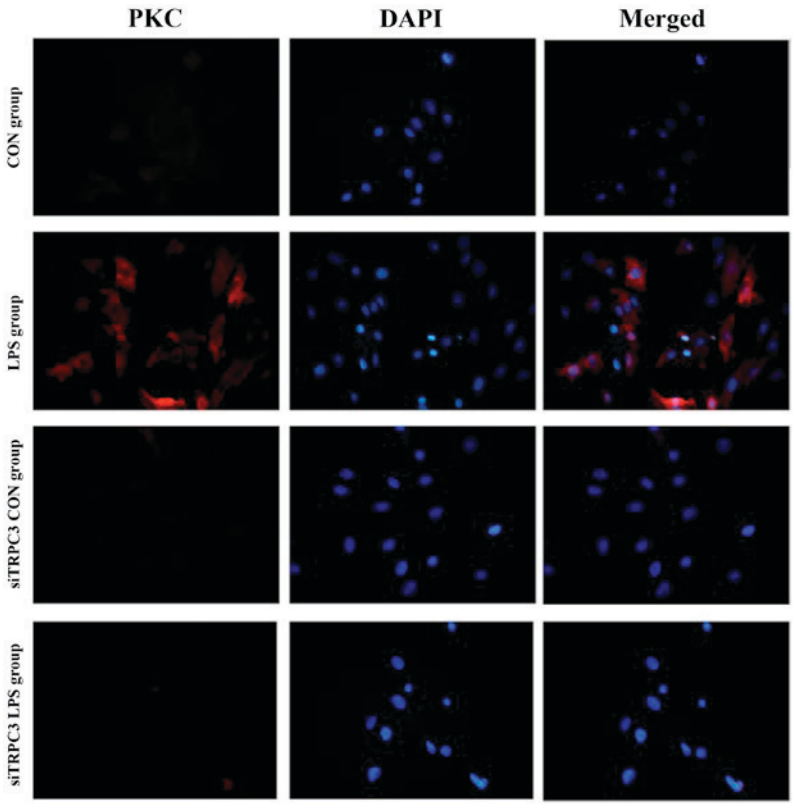

Figure 6. Expression of PKC protein with and without TRPC3-siRNA transfection in murine uterine smooth muscle cells. CON, untransfected non-infected with LPS; LPS, infected with LPS; siTRPC3 CON, transfected with siTRPC3 and non-infected with LPS; siTRPC3 LPS, transfected with siTRPC3 and infected with LPS. PKC, protein kinase C; TRPC3, canonical transient receptor potential 3; siRNA, small interfering RNA; LPS, lipopolysaccharides; CON, control.

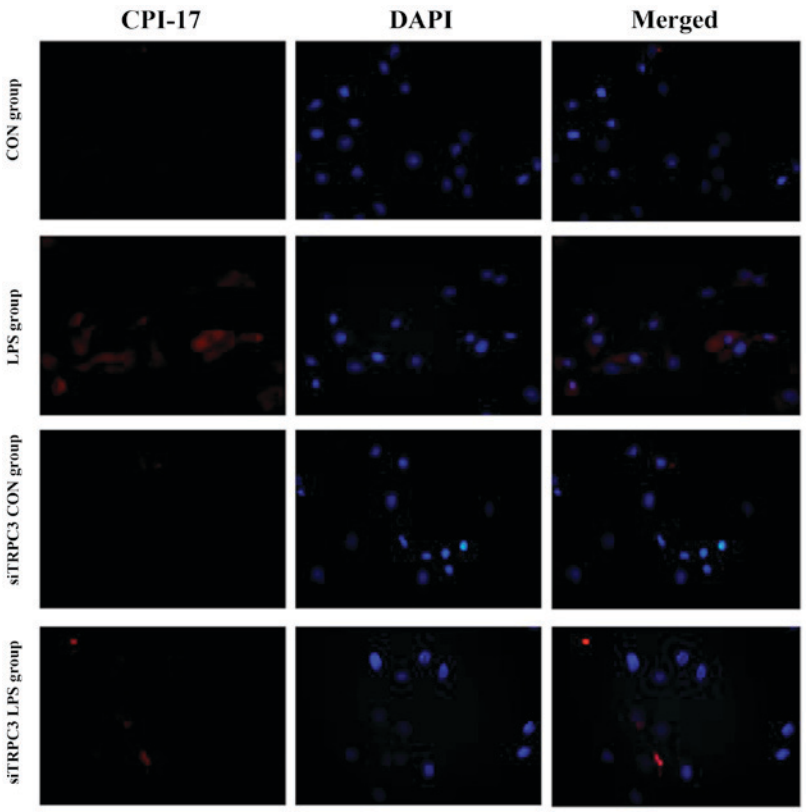

Figure 7. Expression of CPI-17 protein with and without TRPC3-siRNA transfection in murine uterine smooth muscle cells. CON, untransfected non-infected with LPS; LPS, infected with LPS; siTRPC3 CON, transfected with siTRPC3 and non-infected with LPS; siTRPC3 LPS, transfected with siTRPC3 and infected with LPS. CPI-17, C-kinase-activated protein phosphatase-1 inhibitor; TRPC3, canonical transient receptor potential 3; siRNA, small interfering RNA; LPS, lipopolysaccharides; CON, control.

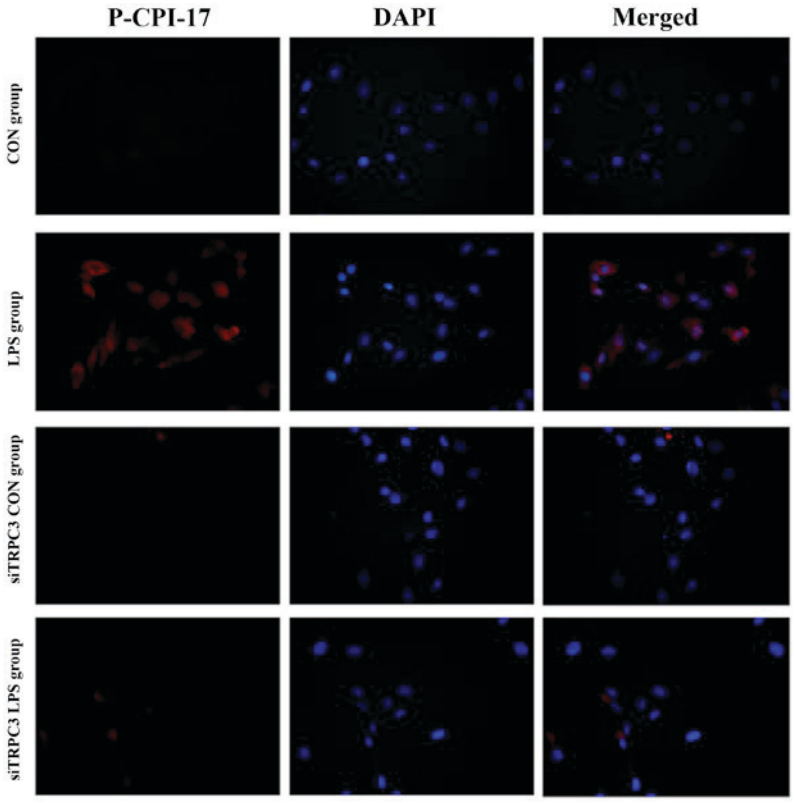

Figure 8. Expression of P-CPI-17 protein with and without TRPC3-siRNA transfection murine uterine smooth muscle cells. CON, untransfected non-infected with LPS; LPS, infected with LPS; siTRPC3 CON, transfected with siTRPC3 and non-infected with LPS; siTRPC3 LPS, transfected with siTRPC3 and infected with LPS. P-, phosphorylated; CPI-17, C-kinase-activated protein phosphatase-1 inhibitor; TRPC3, canonical transient receptor potential 3; siRNA, small interfering RNA; LPS, lipopolysaccharides; CON, control.

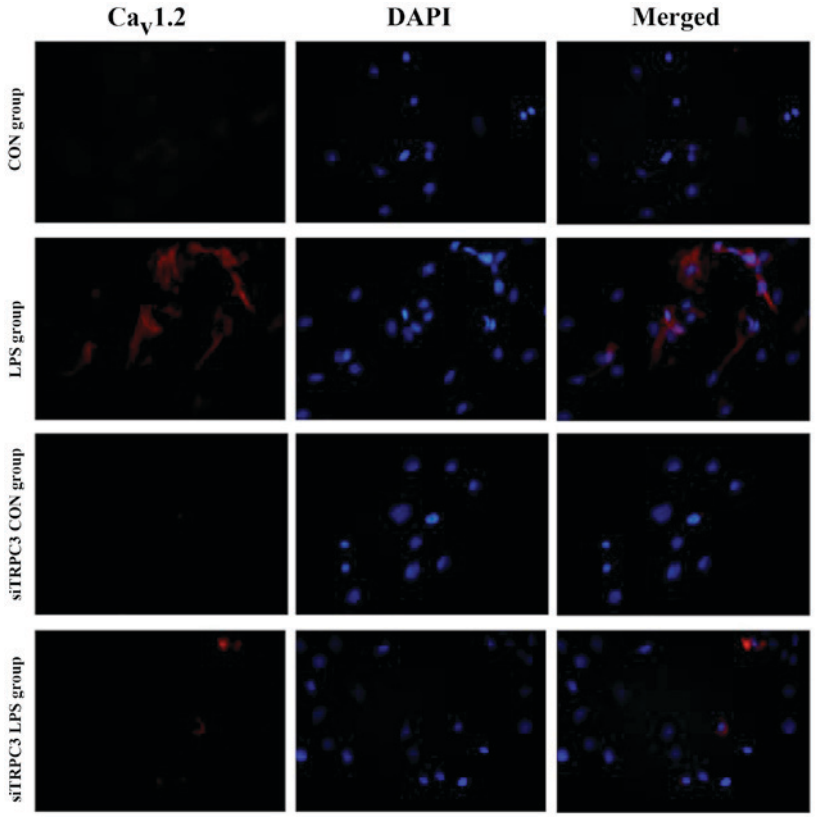

Figure 9. Expression of $Ca_v1.2$ protein with and without TRPC3-siRNA transfection in murine uterine smooth muscle cells. CON, untransfected non-infected with LPS; LPS, infected with LPS; siTRPC3 CON, transfected with siTRPC3 and non-infected with LPS; siTRPC3 LPS, transfected with siTRPC3 and infected with LPS. TRPC3, canonical transient receptor potential 3; siRNA, small interfering RNA; LPS, lipopolysaccharides; CON, control.

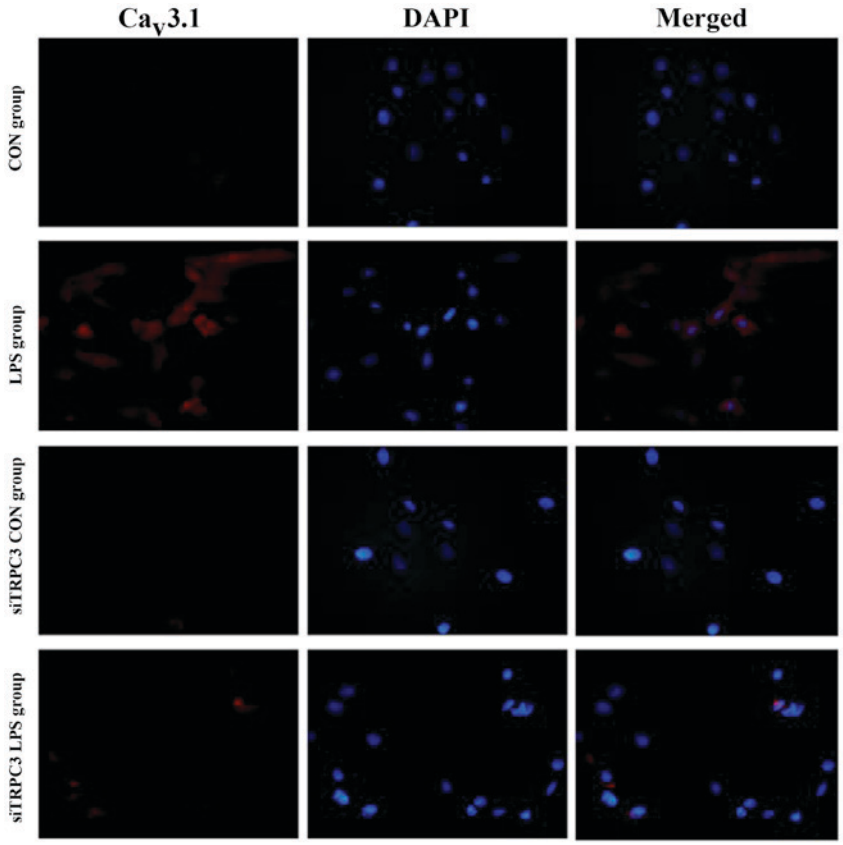

Figure 10. Expression of $Ca_v3.1$ protein with and without TRPC3-siRNA transfection in murine uterine smooth muscle cells. CON, untransfected non-infected with LPS; LPS, infected with LPS; siTRPC3 CON, transfected with siTRPC3 and non-infected with LPS; siTRPC3 LPS, transfected with siTRPC3 and infected with LPS. TRPC3, canonical transient receptor potential 3; siRNA, small interfering RNA; LPS, lipopolysaccharides; CON, control.

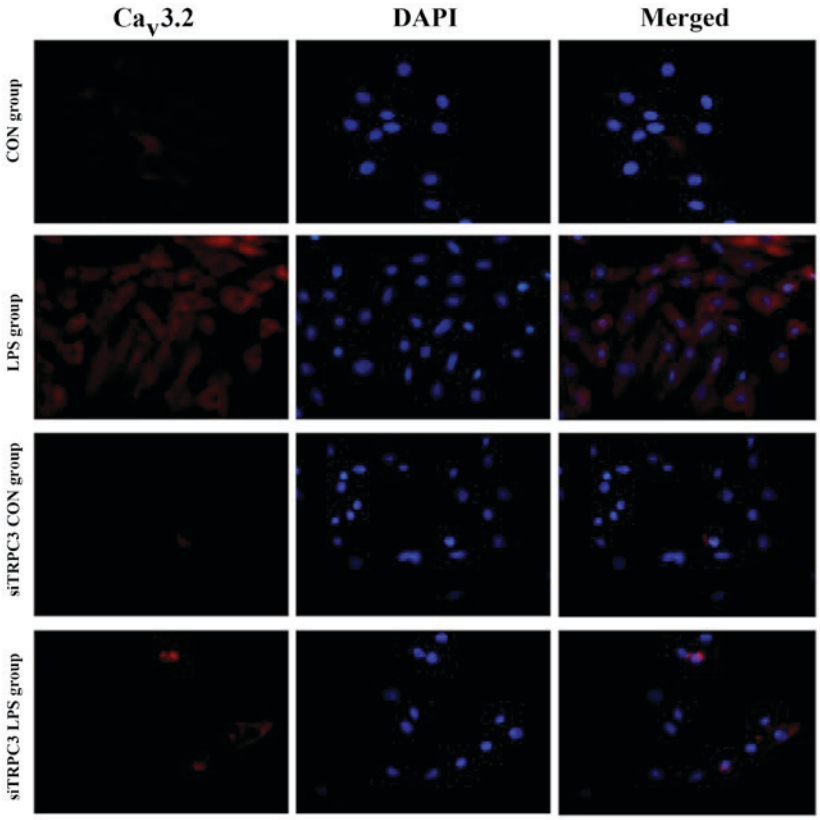

Figure 11. Expression of $Ca_V3.2$ protein with and without TRPC3-siRNA transfection in murine uterine smooth muscle cells. CON, untransfected non-infected with LPS; LPS, infected with LPS; siTRPC3 CON, transfected with siTRPC3 and non-infected with LPS; siTRPC3 LPS, transfected with siTRPC3 and infected with LPS. TRPC3, canonical transient receptor potential 3; siRNA, small interfering RNA; LPS, lipopolysaccharides; CON, control.

and inhibits the calcium sensitization mediated by phosphorylated CPI-17; thus, this pathway reduces the Ca^{2+} sensitivity of smooth muscle cells (15).

A previous study identified that there were no significant differences in CPI-17 mRNA and protein expression levels between the unfertilized group, full-term delivery without labor onset group and full-term delivery with labor onset group; however, phosphorylated CPI-17 protein in uterine smooth muscle tissue was lower in the unfertilized group compared with the full-term delivery without labor onset group and full-term delivery with labor onset group (16,17). Furthermore phosphorylated CPI-17 protein levels were significantly higher in the full-term delivery with labor onset group than in the full-term delivery without labor onset group. These differences may be explained by the following: During pregnancy, endothelin-1 and TNF-α (16,17) increase the phosphorylation of CPI-17, compared with the unfertilized group, whereas the high NO in uterine smooth muscle tissue inhibits the effect of phosphorylated CPI-17; during late pregnancy, prostaglandin and oxytocin levels in uterine smooth muscle gradually increase, and PKCβ expression is upregulated, which induces CPI-17 phosphorylation at Thr-38 and increases the expression level and activity of phosphorylated CPI-17; prior to parturition, the NO level sharply decreases (13), and the inhibitory effect on phosphorylated CPI-17 is reduced; therefore, phosphorylated CPI-17 levels increase rapidly, which inhibits the activity of MLCP, resulting in increased phosphorylation of MLC20. Phosphorylated MLC20 causes myosin head swinging and binding with actin, which induces uterine smooth muscle contraction and triggers parturition.

Although PKC and Rho kinase can act on CPI-17, CPI-17 activation-induced contraction is only inhibited by GF109203X, a PKC inhibitor; other inhibitors of contraction-regulating kinases, such as the Rho kinase inhibitor Y-27632, exert few inhibitory effects in rat aortic smooth muscle cells and pulmonary arterial endothelial cells (18-20). This evidence indicates that PKC is the main regulator of CPI-17 and uterine smooth muscle contraction.

PKC, initially discovered by Nishizuka in 1977, is a family of phospholipid-dependent Ca^{2+}-activated protein serine/threonine kinases, and is an important component in cell signal transduction pathways (21). PKC proteins include two domains: The catalytic domain located in the C-terminal; and the regulatory domain located in the N-terminal. In the regulatory domain, there is an amino acid sequence with similarity to PKC substrates, which acts as a false substrate sequence. PKC is commonly present in the cytoplasm in an inactive form, where the substrate cannot enter the catalytic domain due to the interaction with the false substrate sequence (22). When extracellular signals cause PLC activation, IP_3 is produced by hydrolysis of PIP_2. IP_3 subsequently promotes Ca^{2+} release from the ER, which increases the cytoplasmic Ca^{2+} concentration and activates PKC. DAG promotes PKC to translocate from the cytoplasm to the cell membrane. Under physiological conditions, PKC can only be activated by binding with the cell membrane (23).

The administration of a nonspecific PKC inhibitor to full-term pregnant women reduced smooth muscle

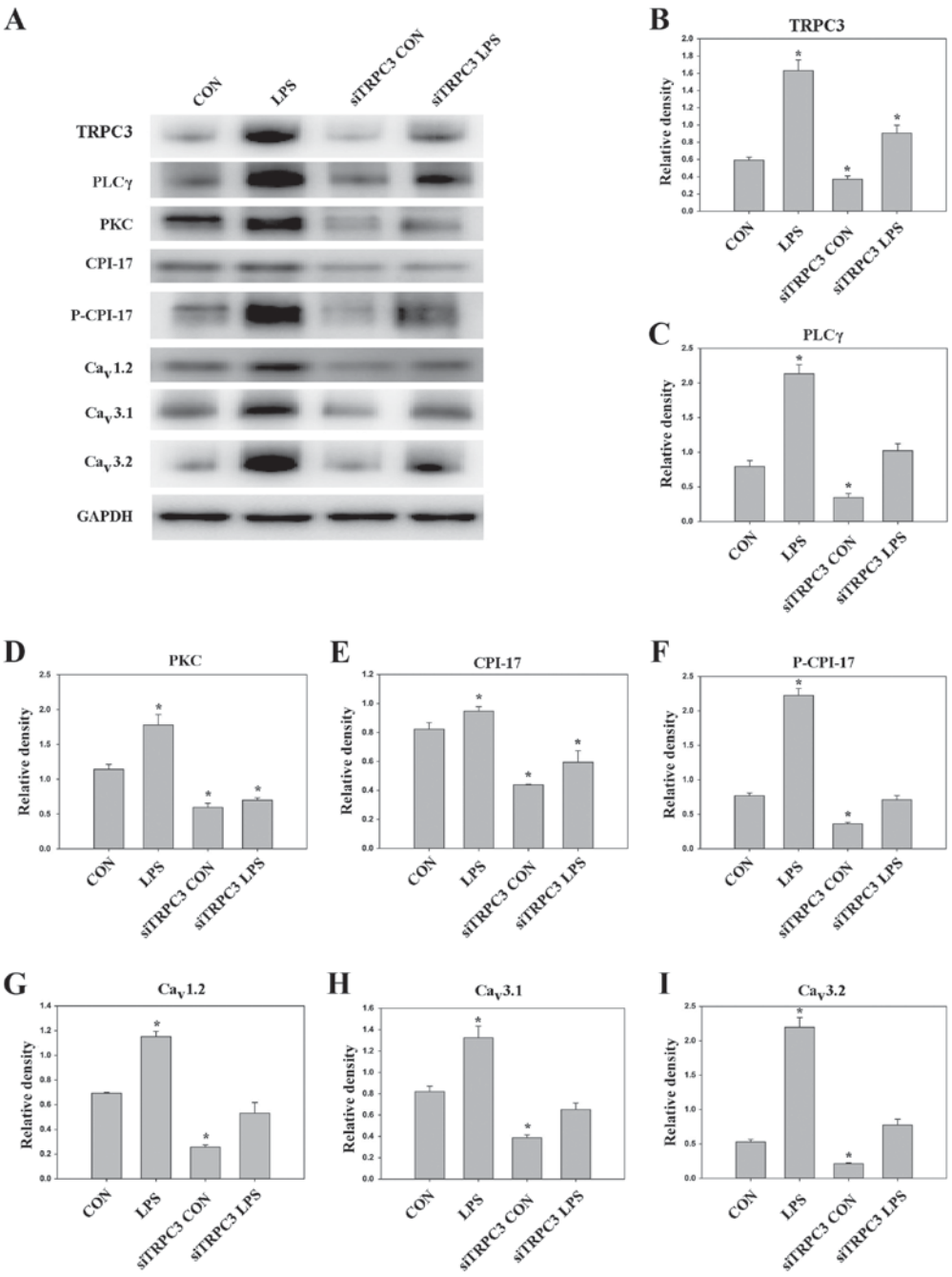

Figure 12. (A) Western blot analysis of proteins in mouse myometrial smooth muscle cells with and without TRPC3-siRNA transfection. GAPDH was used as the loading control. Quantified protein expression levels of (B) TRPC3, (C) PLCγ, (D) PKC, (E) CPI-17, (F) P-CPI-17, (G) $Ca_v1.2$, (H) $Ca_v3.1$ and (I) $Ca_v3.2$. Data are presented as the mean ± standard error. *P<0.05 vs. CON group. CON, untransfected non-infected with LPS; LPS, infected with LPS; siTRPC3 CON, transfected with siTRPC3 and non-infected with LPS; siTRPC3 LPS, transfected with siTRPC3 and infected with LPS. TRPC3, canonical transient receptor potential 3; siRNA, small interfering RNA; PLC, phospholipase C; PKC, protein kinase C; CPI-17, C-kinase-activated protein phosphatase-1 inhibitor; LPS, lipopolysaccharides; CON, control.

contraction by 50-87%, indicating that PKC has some effect on smooth muscle contraction (23). There are 12 subtypes of PKC (23), with varied distribution among different tissues. PKCα, β, γ, δ, z and ι are expressed in the uterine smooth muscle (24). However, it remains unclear which PKC subtype or subtypes contribute to the regulation of uterine smooth muscle contraction. Sakai *et al* (25,26) demonstrated that a PKCβ-specific antagonist inhibits calcium sensitization mediated by phorbol 12,13-dibutyrate, and causes uterine smooth muscle relaxation. These studies suggest that PKCβ subtypes may serve an important role in regulating uterine smooth muscle contraction.

PKCβ mRNA and protein were expressed in the uterine smooth muscle of the full term, preterm group, and full term entry group as detected by RT-qPCR and western blot analyses. PKCβ mRNA and protein expression levels in the full-term delivery with labor onset group were significantly increased compared with the non-pregnant group and full-term delivery without labor onset group, and the expression level in full-term delivery with labor onset group was obviously higher than that

of without labor onset group (5,17,27). This difference may be explained by the observation that prostaglandins, oxytocin receptors and endothelin can stimulate the expression of PKCβ (5,17,27). As the pregnancy continues, the expression of prostaglandins and oxytocin receptor is increased and reaches a peak prior to the parturition. These substances activate specific PLC on uterine smooth muscle cell membrane, which then hydrolyzes PIP_2 to IP_3 and DAG. IP_3 promotes the release of ER Ca^{2+} and increases the cytoplasmic Ca^{2+}. Ca^{2+} binding to the regulatory domain of PKCβ area can induce conformational changes, and rapidly increase the catalytic activity of the protein. DAG stimulates translocation of PKCβ from the cytoplasm to the cell membrane, which then triggers a downstream signaling cascade that increases MLC20 phosphorylation in the uterine smooth muscle cells to enhance uterine smooth muscle contraction and induce labor.

In summary, the results of the current study suggest that PKCβ/CPI-17 signaling may serve a role in triggering labor, and it is suggested that inhibiting this pathway may suppress birth; however, the pathway regulatory mechanism and its interaction with other signaling pathways remains unclear. Further investigation of PKCβ/CPI-17 signaling will aid in the clarification of the mechanism and may potentially provide novel treatments for the prevention of preterm delivery.

Acknowledgements

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