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Enhanced expression of transient receptor potential channel 3 in uterine smooth muscle tissues of lipopolysaccharide-induced preterm delivery mice

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ABSTRACT

Objective(s): We aimed to investigate the influence of transient receptor potential channel 3 (TRPC3) on lipopolysaccharide-induced (LPS) preterm delivery mice.

Materials and Methods: Mice were randomly assigned to the four groups: an unpregnant group, a mid-pregnancy group (E15), a term delivery group, and an LPS-induced preterm delivery group (intraperitoneal injection LPS at 15 days). Uterine smooth muscles were obtained through caesarean section; TRPC3 expression was measured by real-time PCR, western blotting, and immunohistochemistry. A specific inhibitor of TRPC3 (SKF96365) was injected into the LPS-induced preterm delivery group to determine whether the delivery interval was prolonged.

Results: TRPC3 was primarily expressed in the uterine smooth muscle layer. In addition, the LPS-induced preterm delivery group had an obviously higher expression level of TRPC3 mRNA and protein compared with the unpregnant and E15 groups, which were close to term delivery. More importantly, SKF96365 prolongs the delivery interval of LPS-induced preterm delivery mice.

Conclusion: Enhanced expression of TRPC3 may be associated with LPS-induced preterm delivery in mice. The specific inhibitor of TRPC3 (SKF96365) may be helpful for clinical treatment of preterm delivery.

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Introduction

Preterm delivery is the main cause of perinatal infant mortality and morbidity. In China, 19.7% of patients in our neonatology department are preterm; preterm birth accounts for greater than 70% of cases of perinatal death (1). Survivals may experience serious complications such as respiratory distress syndrome, cerebral palsy, and intracranial hemorrhage, up to 25% of whom have neurologic and cognitive sequelae (1). Infection during pregnancy is the main cause of spontaneous preterm birth, which may lead to a higher incidence of neurologic sequelae. However, there is a lack of effective measures for prevention and treatment of preterm delivery, primarily because the pathophysiology of labor has not been fully elucidated.

Parkington *et al* (2) reported that uterine contractility was determined by the concentration of calcium in muscle cells. Before uterine contractions, cytoplasmic calcium increases significantly. Recently, many studies have investigated the effect of L-type calcium channels on preterm delivery. However, the studies cannot fully explain preterm delivery (3).

Transient receptor potential (TRP) channels are a family of generally nonselective cation channels that are activated and regulated by a wide variety of stimuli (4-7), and play significant roles in cellular calcium homeostasis. They are involved in many physiopathologic processes, such as the formation of excitatory synapses (8, 9), muscle cell proliferation (10), cancer (11, 12), kidney disease (13), and platelet activation (14). TRP canonical type 3 (TRPC3) is widely expressed in the uterine smooth muscle of mammals (15). Upregulation of TRPC3 in the human myometrium has been suggested to play a role in uterine contractions during pregnancy and labor (16). We hypothesize that TRPC3 is likely to be involved in preterm labor and delivery, and have focused our study on mice uterine smooth muscle.

Materials and Methods

Subjects

An infectious mouse model of intrauterine inflammation was used, as previously reported (17-19). Twelve-week-old female C57BL/6 mice weighing 22-25

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Table 1. Primer sequences used for real-time PCR

Gene	Gene ID	Primers	Sequences(5′→3′)	Sequence length	Annealing temperature
TRPC3	22065	Forward	ATCTGGAACTGGGCATGGGTA	118bp	60°C
		Reverse	TGATATCGTGTTGGCTGATTGAGAA		
GAPDH	14433	Forward	TGTGTCCGTCGTGGATCTGA	150bp	60 °C
		Reverse	TTGCTGTTGAAGTCGCAGGAG		

g were purchased from the Animal Center laboratory of the China Medical University (Shenyang, China) and used for this study. This strain of mice has a gestational period of 19-21 days. We defined the preterm period as 70% of gestation which would be embryonic day 15 (E15) in this strain of mice (20). Lipopolysaccharide (LPS) (Sigma, St Louis, MO, USA; 50 μg in 100 μl sterile saline) was infused on E15 (16-19), with care not to inject into the amniotic cavity. This study was approved by the Ethics Committees of Shengjing Hospital of China Medical University.

The mice were fed and housed in a standard laboratory environment for one week, for adaptation prior to the experiment. On day 1, mice were housed together in a cage, with a female-to-male ratio of 2:1. Vaginal plugs were checked on the morning of the day 2. When positive for a vaginal plug, the mouse was listed as gestational day 0. Pregnancy was confirmed on gestational day 7 and 12. Healthy pregnant mice were selected for the experiments. Delivery was defined as the delivery of at least one pup. After the mice were placed under deep anesthesia, uterine smooth muscle was obtained at caesarean section when the mice were unpregnant (n=12), at mid-pregnancy (15 days, n=12), or at term (n=12), and LPS intraperitoneal injection at 15 days (n=12). Real-time PCR, Western blotting, and immunohistochemistry were employed to measure TRPC3 expression, as outlined below.

Total RNA extraction, reverse transcription of RNA, and real-time PCR

Total RNA from 100 mg mouse uterine tissue was extracted by the TRIzol reagent (Invitrogen Life Technologies, Nanjing, China) according to the manufacturer's instructions. RNA concentration and purity were quantified in a GenQuant RNA/DNA calculator (Amersham-Pharmacia Biotech, Cambridge, UK). Deoxyribonuclease (DNase) treatment was carried out using DNase I (Invitrogen Life Technologies, Carlsbad, CA, USA). Two micrograms of total mouse uterine RNA samples were reverse-transcribed with TaKaRa (Bio Company, Dalian, China) according to the manufacturer's instructions. Reverse transcription and PCR were performed according to the manufacturer's instructions.

Real-time PCR analysis was then performed to examine TRPC3 mRNA in mice uterine smooth muscle using a Rotor-Gene Q sequence detector (Applied Biosystems, Berlin, Germany). Real-time PCRs were carried out using a SYBR® Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA). The sequences of primers specific to TRPC3 and GAPDH are listed in Table 1.

The PCRs were carried out in triplicate, in a final volume of 25 μl with 2.5 μl (100 ng) cDNA, SYBR® Green Realtime PCR Master Mix 12.5 μl, and 0.5 μl sense and antisense primer mixture (10 mmol). The specificity of the amplification products was confirmed by melting curve analysis. PCR was performed for 60 sec at 95 °C followed by 40 cycles of 15 sec denaturation at 95 °C and 60 sec annealing 60 °C. The quantity of cDNA for each experimental gene was normalized to the quantity of GAPDH cDNA in each sample. Relative expression was determined by using the ΔΔCt (threshold cycle) method (19).

Western blotting

Protein extracts were prepared from mice uterine smooth muscle by homogenization with modified radioimmunoprecipitation assay lysis buffer (50 mmol/l Tris-HCl, 150 mmol/l NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mmol/l EDTA, 0.1% SDS) supplement with protease inhibitor cocktail in tablets (Roche Diagnostics, Basel, Switzerland), and proteins were isolated in an extraction buffer (125 mmol/l Tris-HCl pH 8.0, 2 mmol/l CaCl2, 1.4% Triton X-100 (v/v) and protease inhibitor cocktail in Tablets). Protein concentrations were quantified with a bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific Inc, Rochester, NY, USA). Protein samples (50 mg) and a prestained protein ladder were migrated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on a polyvinylidene fluorid (PVDF) membrane (Amersham Biosciences, Piscataway, NJ, USA). Membranes were blocked with 5% non-fat dried milk in Tris-buffered saline (TBS) with 0.15% Tween-20 and incubated with the primary antibody. The following primary antibodies were used: rabbit anti-mouse TRPC3 monoclonal antibodies (1:2000; Cat.ab75171, Abcam, Ltd., Hong Kong, China), and rabbit polyclonal to α-tubulin antibody (1:10000; Cat. ab125267, Abcam). α-Tubulin expression served as a loading control. Primary antibody binding was detected using the following secondary antibody: anti-rabbit IgG and anti-mouse IgG antibody conjugated to horseradish perioxidase (1:10000; Santa Cruz Biotechnologies, USA). Detection was achieved with an ECL chemiluminescence kit (Amersham Bioscience) according to the protocol using radiographic films (Kodak, China). Image-J Software was used to quantify the level of protein expression.

Immunohistochemistry

Sections were cut at 4 µm and mounted onto poly-L-lysine coated glass slides. Sections were dried in an oven overnight at 37 °C and dewaxed in xylene, rehydrated by passing through descending dilutions of ethanol (70%-100%), and washed in phosphate-buffered saline (PBS). Sections were treated with 3% hydrogen peroxide for 10 min in order to block endogenous peroxidase activity before being rinsed in PBS for 3× 5 min cycles. Slides were treated with normal goat serum diluted in PBS in order to prevent nonspecific binding before being incubated in a moist chamber for 30 min in 37 °C oven. Sections were incubated in a moist chamber for 24 hr at 4 °C with TRPC3 primary antibody (rabbit anti-mouse TRPC3, 1:800, Cat.ab75171, Abcam). Slides were washed in PBS for 3× 5 min cycles and then incubated further with biotinylated anti-rabbit secondary antibody in PBS (1:200, Vector Labs, Burlingame, CA, USA) for 30 min at 37 °C. Then, 3× 5 min washes in PBS were performed and slides were incubated with an streptomycin avidia-biotin-peroxidase complex. After a 30 min incubation at 37 °C, sections were washed three times in PBS. For color visualization of the primary antigen-antibody complex, peroxidase substrate solution (DAB stain kit, Vector Labs, Burlingame, CA, USA) was applied to all sections followed by incubation in a dark and moist chamber for 5 min. After washing in tap water, a light hematoxylin counterstain was used and sections were dehydrated through graded concentrations of ethanol and cleared through three changes of xylene. After mounting media and coverslips, the sections were examined by light microscopy. Negative controls were performed by omitting the primary antibodies; the specificity of these antibodies has been reported in previous studies (21-25).

Immunohistochemistry Semi-quantitation

Semi-quantitative immunohistochemical analysis of TRPC3 was performed according to a previously published method (26). Briefly, 10 fields were randomly selected and expression in 100 cells/field were evaluated using high-power (200× or 400×) microscopy. Three investigators (Dongming Zheng, Lijuan Zhang and Caixia Liu) separately evaluated the staining in a blinded manner. The mean values of each investigator's scores were used for analysis. Specifically, staining intensity was rated on a scale of 0-3 (0=negative, 1=weak, 2=moderate, and 3=strong). No positive cell was scored as 0, 1%-10% of positive cells as 1, 11%-50% as 2, 51%-80% as 3, and 81-100% as 4. The intensity score was multiplied by the percentage of positive cell score to obtain a final number for further statistical analysis.

Using the specific inhibitor of TRPC3 (SKF96365)

Thirty min after injecting 50 µg of LPS, we injected the specific inhibitor of TRPC3 (SKF96365) intraperitoneally in mice. SKF96365 was purchased from Sigma (No. S7809). Twenty micrograms, 40 µg, and 80 µg were infused, respectively. Delivery intervals were recorded.

Statistical analysis

Results of real-time PCR experiments, Western blotting analysis and immunohistochemistry were analyzed for statistical significance by the student's T test with GraphPad Prism version 5.0 for Windows (GraphPad Software). A P-value of less than 0.05 was considered statistically significant. ns: non significant difference, * P<0.05, ** P<0.01, *** P<0.001.

Results

mRNA expression of TRPC3 by real-time PCR

TRPC3 mRNA transcripts were detected in uterine smooth muscle in unpregnant mice, those during mid-pregnancy (E15), term delivery, and LPS-induced preterm delivery. We found that TRPC3 mRNA expression increased with gestational days. mRNA expression level in the LPS-induced preterm delivery group was close to that of term delivery, more than levels observed while unpregnant and at E15 (Figure 1).

Protein expression of TRPC3 by Western blotting

Using western blotting, TRPC3 protein was detected in the uterine smooth muscle in unpregnant mice, those during mid-pregnancy (E15), term delivery, and LPS-induced preterm delivery. Figure 2 shows the TRPC3 protein expression level to be increased with

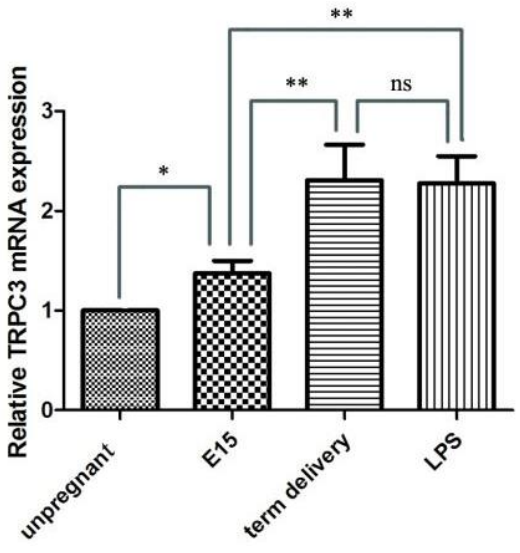

Figure 1. Expression of TRPC3 mRNA in mice uterine smooth muscle. Real-time PCR was performed on samples, with the results expressed as the average ± standard of the TRPC3/GAPDH ratio of four group tissue samples. (i) The P-value of unpregnant vs. mid-pregnancy (E15) is 0.013 (*). (ii) The P-value of E15 vs. term delivery is 0.006 (**). (iii) The P-value of term delivery vs. intraperitoneal injected of LPS at 15 d (LPS) is 0.212 (ns). (iv) The P-value of E15 vs. LPS is 0.005 (**)

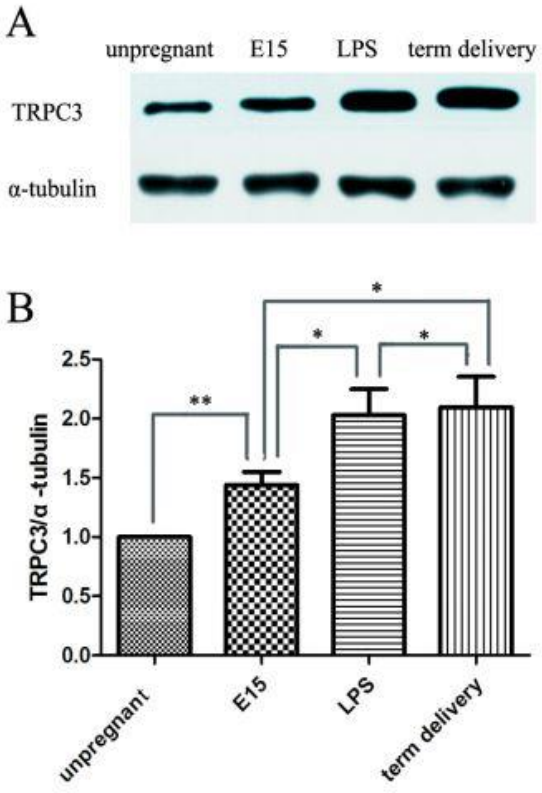

Figure 2. Expression of TRPC3 protein in mice uterine tissue. Western blotting was used on the four group tissue samples (A), with the results expressed as the average ± standard error of the mean of the TRPC3/α-tubulin ratio (B). (i) The P-value of unpregnant vs. E15 is 0.003 (**). (ii) The P-value of E15 vs. term delivery is 0.028 (*). (iii) The P-value of term delivery vs. LPS is 0.047 (*). (iv) The P-value of E15 vs. LPS is 0.049 (*)

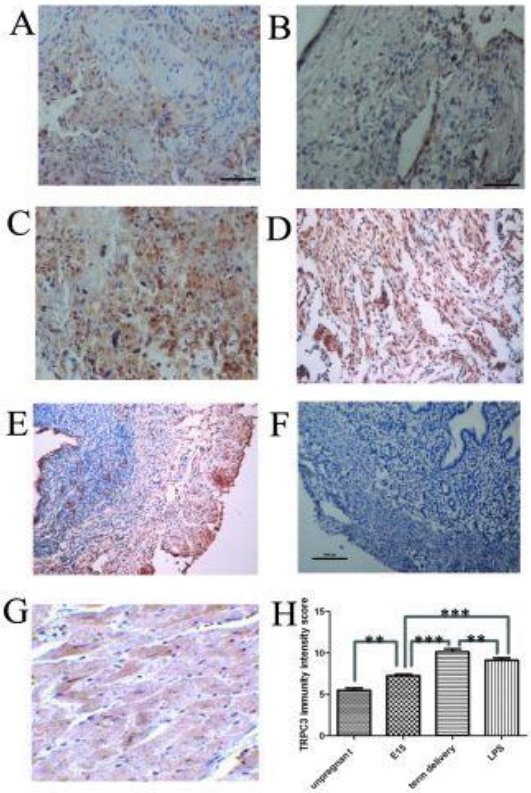

Figure 3. Immunohistochemistry of TRPC3 expression in mice uterine smooth muscle of unpregnant (A, 400×), E15 (B, 400×), term delivery (C, 400×), LPS (D, 400×), negative control (F, 200×), and positive control (G, 400×) groups. Panel H shows TRPC3 immunity intensity score semiquantitation. Term delivery (C) has higher expression of TRPC3 compared to unpregnant (A) and E15 (B) mice. LPS (D) has the same intensity expression of TRPC3, compared with term delivery. E (200×) shows that TRPC3 mainly expressed in the uterine smooth muscle layer and blood vessels. Negative controls were performed by omitting the primary antibodies (F). Mice cardia tissues were used as a positive control (G)

gestational days. And the protein expression level of LPS-induced preterm delivery was close to that of term delivery, more than the levels observed during the unpregnant state and E15 (Figure 2).

Protein expression of TRPC3 by immunohistochemistry

Immunohistochemistry was performed on uterine smooth muscle. TRPC3 was primarily expressed in the uterine smooth muscle layer and blood vessels. High TRPC3 expression was detected in term delivery and LPS-induced preterm delivery groups. On the contrary, low expression was detected in the unpregnant and E15 groups (Figure 3).

Delivery interval of LPS-induced mice prolonged by SKF96365

SKF96365 is the specific inhibitor of TRPC3. Thirty min after injecting 50 μg of LPS, we injected SKF96365 intraperitoneally in mice. Twenty micrograms, 40 μg and 80 μg were infused, respectively. SKF96365 can prolong the delivery interval of LPS-induced preterm delivery (Figure 4).

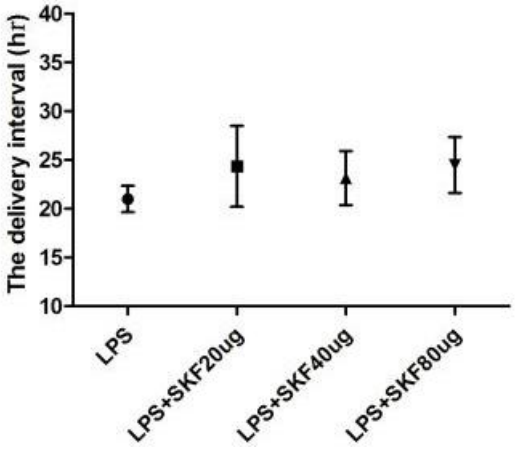

Figure 4. The specific inhibitor of TRPC3 (SKF96365) can prolong the delivery interval of LPS-induced mice. The delivery interval is between the time of injecting LPS and the beginning of labor. (i) The P-value of LPS vs. LPS+SKF96365 (20 ug) is 0.019 (*). (ii) The P-value of LPS+SKF96365 (20 ug) vs. LPS+SKF96365 (40 ug) is 0.431 (ns). (iii) The P-value of LPS+SKF96365 (20 ug) vs. LPS+SKF96365 (80 ug) is 0.475 (ns)

Discussion

Preterm delivery is considered to be an early stage of the delivery process; the uterus is changing from the resting to activated state. Generally, it is believed that calcium participates in uterine smooth muscle contractions during labor (2, 27). Nifedipine, the inhibitor of L-type calcium channels, has been used to prevent preterm birth in the clinical setting (28). However, clinical treatments showed that nearly half of preterm patients responded negatively to it (29, 30). Studies conducted by Mori *et al* (31) and Liu *et al* (32) suggested that increased cytoplasmic calcium in preterm patients resulted from calcium influx via L-type calcium and other calcium-permeable channels. Our study has focused on increased TRPC3 expression in LPS-induced preterm delivery mice.

TRPC3 is the putative capacitative calcium entry channel that responds to contractant-generated signals and intracellular calcium storage depletion. Dalrymple *et al* (33) showed a significant increase in TRPC3 protein on the stretch-induced effects in human myometrial smooth muscle cells (PHM1-41 cells). Also, TRPC3 mRNA was detected in uterine smooth muscle in pregnant women (34). Consistent with these findings, we have detected protein and mRNA of TRPC3 in mice uterine smooth muscle during the unpregnant state, mid-pregnancy (E15), term delivery, and LPS-induced preterm delivery.

Our present data support the important role of TRPC3 in the uterine smooth muscle of pregnant mice. We demonstrate a significant enhancement in TRPC3 expression both in the RNA and protein levels in mice uterine smooth muscle during the unpregnant state, mid-pregnancy (E15), term delivery, and LPS-induced preterm delivery. Senadheera *et al* (35) demonstrated that TRPC3 was important in regulating uterine contraction in rats, with the mechanism augmented in pregnancy. Our study also indicates that upregulated TRPC3 may play an important role in LPS-induced preterm delivery. By intraperitoneally injecting the specific inhibitor of TRPC3 (SKF96365) into LPS-induced preterm delivery mice, we found that the delivery interval was obviously prolonged.

We found that TRPC3 mRNA and protein expression increased as pregnancy progressed,which is in agreement with the report by Dalrymple *et al* (33). We found that TRPC3 mRNA and protein of LPS-induced preterm delivery was close to the level of term delivery, and obviously higher than cesarean section at pregnancy of 15 days, which was inconsistent with the results of Babich *et al* (36). Dalrymple *et al* (37) declared that TRPC3 in term labor women was higher than that of term nonlabor women, and was much higher than in unpregnant women. The above results suggest that TRPC3 may play a role in the onset of preterm delivery.

Conclusion

In LPS-induced preterm delivery in mice, TRPC3 expression enhances on both the RNA and protein levels. In addition, the delivery interval after LPS injection can be prolonged by the specific inhibitor of TRPC3 (SKF96365). TRPC3 has a strong presence in preterm delivery; its reduction may be considered as a novel therapeutic target in the clinical treatment of premature delivery.

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Conflicts of interests

The authors declare that they have no competing interests.

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