

Lipopolysaccharides upregulate calcium concentration in mouse uterine smooth muscle cells through the T-type calcium channels

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Abstract. Infection is a significant cause of preterm birth. Abnormal changes in intracellular calcium signals are the ultimate triggers of early uterine contractions that result in preterm birth. T-type calcium channels play an important role in the pathogenesis of cancer, as well as endocrine and cardiovascular diseases. However, there are limited studies on their role in uterine contractions and parturition. In the present study, mouse uterine smooth muscle cells were isolated and treated with lipopolysaccharides (LPS) to mimic the microenvironment of uterine infection *in vitro* to investigate the role of T-type calcium channels in the process of infection-induced preterm birth. The results from quantitative polymerase chain reaction and western blot analysis showed that LPS significantly induced the expression of the Cav3.1 and Cav3.2 subtypes of T-type calcium channels. Measurements of intracellular calcium concentration showed a significant increase in response to LPS. However, these effects can be reversed by T-type calcium channel blockers. Western blot analysis further indicated that LPS induced the activation of the nuclear factor (NF)- κ B signaling pathway, and endothelin-1 (ET-1) was significantly upregulated, whereas NF- κ B inhibitors significantly inhibited the LPS-induced upregulation of Cav3.1, Cav3.2 and ET-1 expression. In addition, ET-1 directly induced Cav3.1 and Cav3.2 expression, whereas ET-1 antagonists inhibited the LPS-induced upregulation of Cav3.1 and Cav3.2 expression. In conclusion, the present study demonstrates that infection triggers the upregulation of T-type calcium channels and promotes calcium influx. This process relies on the activation of the NF- κ B/ET-1 signaling pathway. The T-type calcium channel is expected to become an effective target for the prevention of infection-induced preterm birth.

Introduction

Preterm birth is an extremely common and problematic complication in perinatal medicine and is one of the leading causes of perinatal mortality (1,2). Infants that survive preterm birth often suffer from health problems, including cerebral palsy, cognitive dysfunction, blindness, hearing loss and respiratory system damage (3,4), which can place a significant responsibility on individuals and families. Clinical studies have shown that 40% of preterm births are caused by infection (5). Accumulating evidence indicates that infection is not only an important reason to induce preterm labor (6,7) but also a potential target for the prevention of preterm birth (8). Therefore, it is important to further explore the pathogenesis of preterm birth caused by infection to reduce the risk of preterm birth and improve the perinatal survival rate.

One of the well-known main clinical manifestations of preterm birth is early contractions of the uterus, and the ultimate factor inducing uterine contraction is the changes of calcium signals in uterine smooth muscle cells (9-11). Voltage-gated calcium-channels (VGCCs) are heterologous multimeric transmembrane proteins located in the cell membrane and they are responsible for transporting extracellular calcium ions into the cells. VGCCs play an important role in the regulation of intracellular calcium signals. The VGCCs of uterine smooth muscles are mainly L- and T-type (12). Previous studies on the inducing factors of obstetric labor have mainly focused on L-type calcium channels. L-type calcium channel inhibitors, such as nifedipine, have been used as one of the first-line drugs in the treatment of premature delivery (13-15). The roles of T-type calcium channels are also extremely extensive, involving areas including tumor development (16-19), and endocrine (20-22) and cardiovascular diseases (23-26). However, there have been limited studies on their role in uterine contractions and labor induction.

In the present study, mouse uterine smooth muscle cells were isolated. Lipopolysaccharides (LPS) were used for cell treatment. Under infectious conditions, the expression of T-type calcium channels and the changes in calcium concentration of uterine smooth muscle cells were measured and the possible mechanisms were explored. The results showed that infection induced the upregulation of T-type calcium channel expression and further increased the concentration of calcium in uterine smooth muscle cells, in which the nuclear factor

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(NF)- κ B/endothelin-1 (ET-1) signaling pathway plays an important role.

Materials and methods

Establishing the mouse model of pregnancy. Twelve-week-old female C57BL/6 mice weighing 22–25 g were purchased from the Animal Center laboratory of the China Medical University (Shenyang, China). The mice were fed and housed in a standard laboratory environment for a week for adaptation prior to the experiment. The maintenance and handling of experimental animals was approved by the Committee on Animal Research and Ethics of the China Medical University. On day 1, the mice were housed together with a female to male ratio of 2:1 in the cage. The vaginal plugs were checked on the morning of the day 2. When positive for a vaginal plug, the mouse was listed as in gestation day 0. Pregnancy was confirmed on gestation days 7 and 12. Healthy pregnant mice were selected for the experiments.

Isolation and culture of mouse uterine smooth muscle cells. Mouse uterine smooth muscle cells were isolated based on the methods used in previous studies (27,28). The uterine tissues of pregnant mice were isolated under sterile conditions. Pre-chilled D-Han's solution (containing 100 U/ml of penicillin-streptomycin) was used to remove impurities. The serosa and endometrium were removed. The intermediate smooth muscle layers were cut into 2–3-mm³ sections. A digestive solution mixture of 0.25% trypsin (Beyotime Institute of Biotechnology, Haimen, China) and 0.25% type I collagenase (Invitrogen Life Technologies, Carlsbad, CA, USA) was added, and digestion proceeded at 37°C for 50 min. Subsequently, 20% fetal bovine serum in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Grand Island, NY, USA) was added to terminate the reaction. The cells were filtered using 200-mesh filters. The supernatant was discarded following centrifugation. After washing with phosphate-buffered saline (PBS), 10% fetal bovine serum in DMEM was added to re-suspend the cells. The cells were subsequently seeded into 6-well plates and placed in the incubator. After 24 h, the cell adherence was observed and the medium was changed. The medium was subsequently changed every 3 days. The cells from the third passage were selected to conduct the experiments. In the LPS group, 2,000 ng/ml LPS was added to treat the cells for 12 h, whereas in the saline group, an equal volume of saline was added. The untreated uterine smooth muscle cells were used as a control group. In the NF- κ B inhibitor group, 100 μ M BAY 11-7028 (Beyotime Institute of Biotechnology) was added prior to treatment with LPS. In the ET-1 treatment group, 50 nM ET-1 (Sigma-Aldrich, St. Louis, MO, USA) was added. In the ET-1 antagonist group, 10 μ M bosentan (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was added.

Immunofluorescence. The third passage of uterine smooth muscle cells were seeded in 6-well plates with cover slips. After the cells became ~100% confluent, 4% paraformaldehyde was applied to fix the cells for 15 min. Once the fixative was discarded, 0.1% Triton X-100 was added until all the cells were covered. The cells were incubated for 30 min at room temperature. Subsequent to blocking the cells with goat serum, α -smooth muscle actin (SMA) antibody (ab40863) with 1:100

dilution (Abcam, Cambridge, MA, USA) was added, and the cells were incubated overnight at 4°C. The cells were washed and fluorescein isothiocyanate-labeled secondary antibody (A0562; Beyotime Institute of Biotechnology) with 1:200 dilution was added, after which the cells were incubated at room temperature for 60 min. 4',6-Diamidino-2-phenylindole (DAPI) was added for nuclear staining. The fluorescence quencher was added dropwise, and the slices were mounted. Images were obtained using a laser scanning confocal microscope (FV1000S-SIM/IX81; Olympus, Tokyo, Japan).

Quantitative polymerase chain reaction (qPCR). TRIzol reagent (Invitrogen Life Technologies) was used to extract the total RNA of the cells in each group. The RNA was reverse transcribed to cDNA using a cDNA first strand synthesis kit (Takara Bio, Inc., Dalian, China), according to the manufacturer's instructions. The upstream and downstream primer sequences of Cav3.1 are 5'-ATAACAGTTCCAGCAATACCACC-3' and 5'-GAATGAGCATCCATCACAAAGT-3', respectively. The length of the amplified fragment was 190 base pairs (bp). The upstream and downstream primer sequences of Cav3.2 are 5'-AGAGCCGTTGGCGTAAGAAG-3' and 5'-GCTGAAGTGGTAATGGTGGTGA-3', respectively. The length of the amplified fragment was 152 bp. The upstream and downstream primer sequences of β -actin are 5'-CTGTGCCCATCTACGAGGGCTAT-3' and 5'-TTTGATGTCACGCACGATTTCC-3', respectively. The length of the amplified fragment was 155 bp. qPCR was conducted using an Exicycler™ 96 Quantitative fluorescence Real-Time PCR System (Bioneer, Daejeon, Korea). The reaction volume was 20 μ l, including 1 μ l cDNA, 0.5 μ l of each upstream and downstream primer, 10 μ l SYBR GREEN mastermix and 8 μ l ddH₂O. The PCR reaction program was 95°C for 10 min followed by 40 cycles of 95°C for 10 sec, 58°C for 20 sec and 72°C for 30 sec; and subsequently 4°C for 5 min.

Western blot analysis. Radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) was used for cell lysis. The bicinchoninic acid method was used for protein quantification and balancing. A total of 40 μ g protein from each group was used for SDS-PAGE. The sample was electronically transferred to a PVDF membrane (Millipore, Bedford, MA, USA) following electrophoresis. After 5% skimmed milk powder was added at room temperature and blocked for 1 h, diluted primary antibody [Cav3.1 (sc-28617) and Cav3.2 (sc-25691), 1:200 dilution; Santa Cruz Biotechnology, Inc.; NF- κ B p65 (WL00666), p-I κ B (WL00020) and ET-1 (WL00138), 1:400 dilution; 1:400 dilution; Wanleibio, Shenyang, China] was added at 4°C and incubated overnight. Subsequently, 1:5,000 diluted horseradish peroxidase-labeled secondary antibody (A0208; Beyotime Institute of Biotechnology) was added and incubated at 37°C for 1 h. An electrochemiluminescence method was used for substrate luminescence. Following exposure, the images were scanned into the computer and grayscale analysis was performed by Image J software (ImageJ Version 1.36b; National Institutes of Health, Bethesda, MD, USA); β -actin was used as an internal reference to analyze the expression level of each protein.

Detection of intracellular calcium ion concentration. The concentration of intracellular calcium ions was measured using

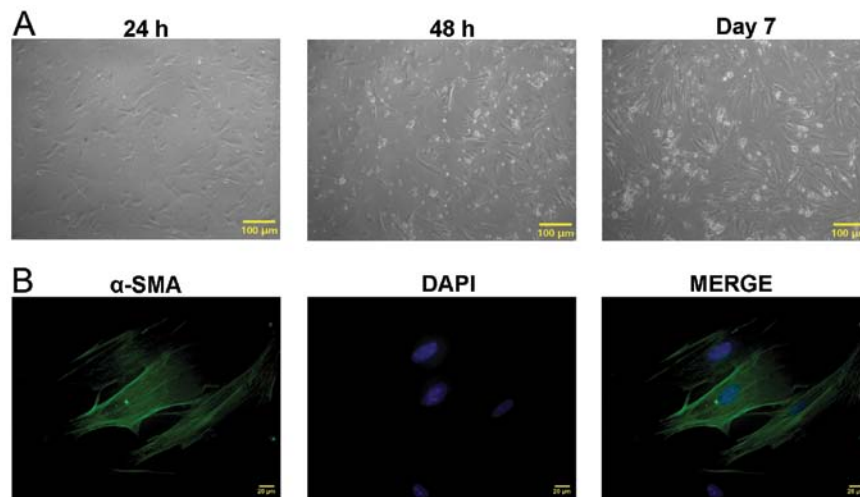


Figure 1. Culture and identification of mouse uterine smooth muscle cells. (A) A trypsin-collagenase I mixed enzyme digestion method was used to isolate uterine smooth muscle cells from pregnant mice. The cell morphology was observed under inverted microscope 24, 48 h and 7 days after inoculation. (B) Immunofluorescence detection of α -SMA expression in mouse uterine smooth muscle cells. Green filamentous α -SMA protein and DAPI stained blue nuclei can be observed within the cells. The representative results are shown in the figure. α -SMA, α -smooth muscle actin; DAPI, 4',6-diamidino-2-phenylindole.

the method of Wang *et al* (29). In the T-type calcium channel inhibitor group, 5 μ M NNC 55-0396 was added 10 min before LPS treatment started (30). After the cells in each group were collected and washed twice with PBS, 1 ml 5 μ M serum-free Fluo-3AM was added and incubated at 37°C in the dark for 30 min. The cells were washed three times and continued to incubate for another 20 min to allow the complete conversion of Fluo-3AM to Fluo-3. Cells were collected and analyzed by a flow cytometer (BD Biosciences, San Jose, CA, USA).

Statistical analysis. The experimental data are presented as the means \pm standard deviation. One-way analysis of variance was used for comparison between the groups. The Bonferroni post-hoc test was used for multiple comparisons. GraphPad Prism 5.0 software (Graph Pad, San Diego, CA, USA) was used for data processing. A value of $P < 0.05$ was considered to represent a statistically significant difference.

Results

Isolation, culture and identification of mouse uterine smooth muscle cells. The uterine tissues of pregnant mice were obtained. Uterine smooth muscle cells were isolated using the trypsin-collagenase I mixed enzyme digestion method (Fig. 1A). After 24 h, the cells had already adhered to the wall of the wells and presented as fusiform or polygonal shapes. The cell clones were visible after 48 h. Cells were fused into pieces after 7 days. The cells from the third passage were collected for α -SMA immunofluorescence staining (Fig. 1B). The results showed green filamentous actin in the cytoplasm, while the nuclei were stained blue with DAPI indicating that mouse uterine smooth muscle cells had been isolated.

LPS induces upregulation of T-type calcium channel subtypes, Cav3.1 and Cav3.2, in mouse uterine smooth muscle cells. Bacterial endotoxin LPS were used to treat uterine smooth muscle cells to mimic an infectious uterine microenvironment *in vitro* (31) to investigate the effect of infection on T-type

calcium channels (Fig. 2). The results showed that after 6 h of LPS treatment, there was a significant increase in Cav3.1 and Cav3.2 mRNA ($P < 0.01$), and the expression increased with the increasing duration of LPS treatment. Western blot analysis of the two proteins showed similar results to the mRNA expression; LPS significantly increased the expression of Cav3.1 and Cav3.2 ($P < 0.01$). Therefore, infection can cause the upregulation of T-type calcium channel expression.

LPS upregulate calcium concentration through T-type calcium channels in uterine smooth muscle cells. Increasing the intracellular calcium concentration can directly cause uterine contractions (32). Therefore, the intracellular calcium concentration of uterine smooth muscle cells was examined to investigate the role of infection and T-type calcium channels in uterine contractions (Fig. 3). The results showed that following LPS treatment, the intracellular calcium concentration increased >3 -fold ($P < 0.01$). However, subsequent to applying the T-type calcium channel inhibitors, the calcium concentration was significantly reduced ($P < 0.01$). As the LPS-induced increases in calcium concentration can be reversed, this indicates that the infection can lead to calcium influx in uterine smooth muscle cells. In this process, T-type calcium channels play an essential role.

LPS upregulates the expression of T-type calcium channels via the NF- κ B signaling pathway. The activation of the NF- κ B signaling pathway was determined to investigate the mechanism of T-type calcium channel upregulation by LPS (Fig. 4). The results showed that compared to the control group, the expression of p-I κ B in the cytoplasm and NF- κ B p65 in the nucleus was significantly higher in the LPS treatment group ($P < 0.01$), indicating that LPS can activate the NF- κ B signaling pathway of mouse uterine smooth muscle cells. Subsequent to applying the NF- κ B inhibitors, the LPS-induced upregulation of Cav3.1 and Cav3.2 was significantly reversed ($P < 0.01$). Therefore, NF- κ B played a role in the LPS-induced upregulation of T-type calcium channel expression.

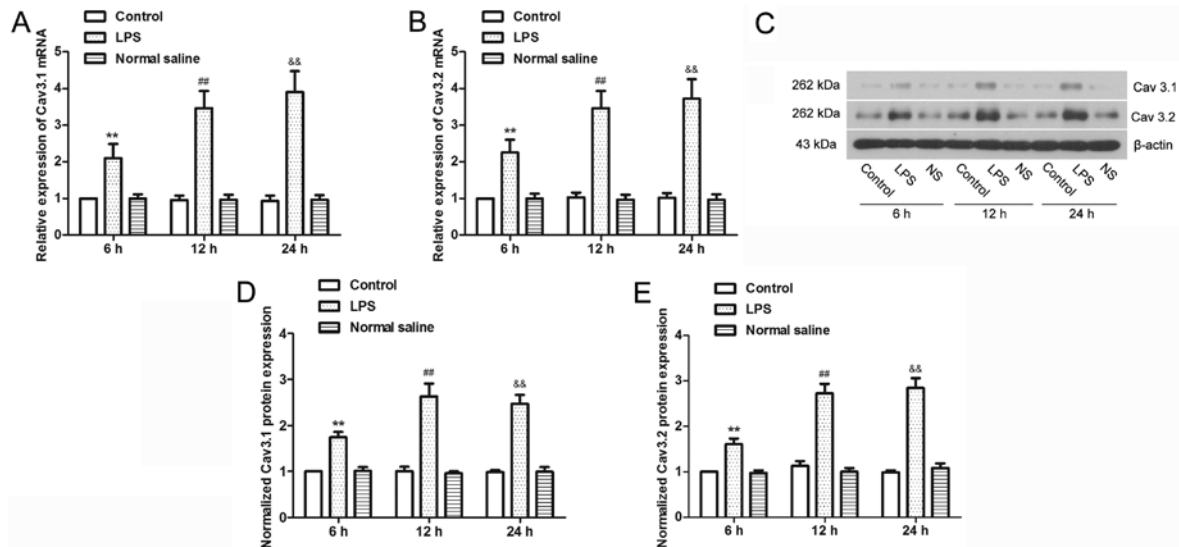


Figure 2. LPS can upregulate the expression of Cav3.1 and Cav3.2 in smooth muscle cells in the uterus. qPCR measured the mRNA expression of (A) Cav3.1 and (B) Cav3.2. (C) Western blot analysis was used to measure the expression of Cav3.1 and Cav3.2 proteins. Typical results from repeated experiments are shown in the figure with β -actin as an internal reference for the analysis of (D) Cav3.1 and (E) Cav3.2 protein levels; $n=3$. The data are expressed as the means \pm standard deviation. ** $P<0.01$ compared to the control group at 6 h; ## $P<0.01$ compared to the control group at 12 h; and && $P<0.01$ compared to the control group at 24 h. LPS, lipopolysaccharides; qPCR, quantitative polymerase chain reaction; NS, normal saline.

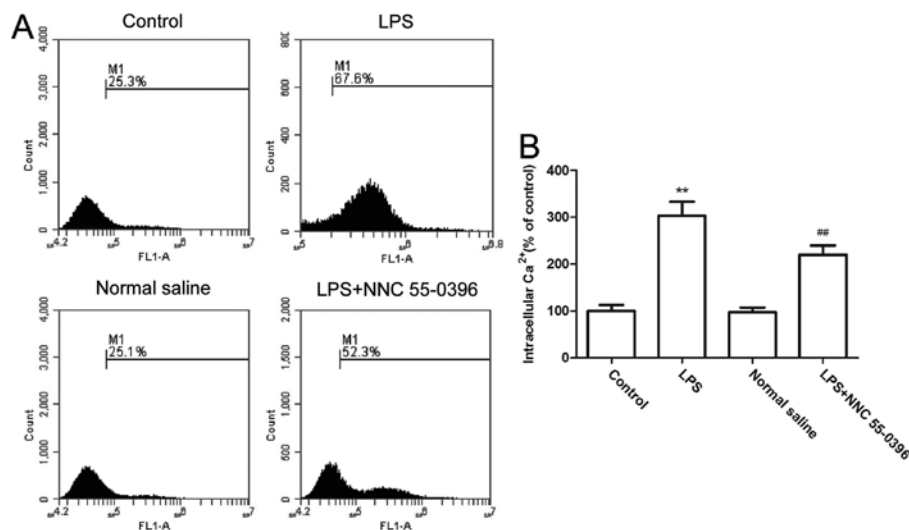


Figure 3. LPS increase calcium concentration in uterine smooth muscle cells. (A) Fluo-3AM tests were used to measure the calcium concentrations in cells of each group. Representative results from repeated experiments of each group are shown in the figure. (B) Relative calcium levels of cells in each group; $n=3$. The data are expressed as the means \pm standard deviation. ** $P<0.01$ compared to the control group; and ## $P<0.01$ compared to the LPS group. LPS, lipopolysaccharides.

ET-1-mediated NF- κ B signaling is involved in the regulation of T-type calcium channels by LPS. ET-1 is an important factor that can induce calcium influx, as well as induce cell contraction (33,34). Therefore, the expression of ET-1 was measured to investigate whether it was involved in the regulation of T-type calcium channels by LPS (Fig. 5). The results showed that the expression of ET-1 was significantly increased subsequent to treatment with LPS ($P<0.01$), indicating that LPS can induce the expression of ET-1 in uterine smooth muscle cells. Further experiments showed that NF- κ B inhibitors reduced the LPS-induced upregulation of ET-1 expression (Fig. 5C and D). Therefore, ET-1 was regulated by NF- κ B. Subsequent to direct treatment of the uterine smooth muscle cells with ET-1, the expression of Cav3.1 and Cav3.2 was upregulated,

while the ET-1 antagonist, bosentan, reversed the effect of LPS on the above two factors (Fig. 5E and F). These results indicate that ET-1 mediates NF- κ B signaling and participates in the regulation of T-type calcium channels by LPS.

Discussion

In the present study, mouse uterine smooth muscle cells were isolated. LPS were applied to treat the cells to mimic the microenvironment of uterine infection *in vitro*. The mechanisms through which an infection affects the T-type calcium channels and intracellular calcium concentration were also explored. The results indicated that LPS significantly upregulated the expression of T-type calcium channel subtypes

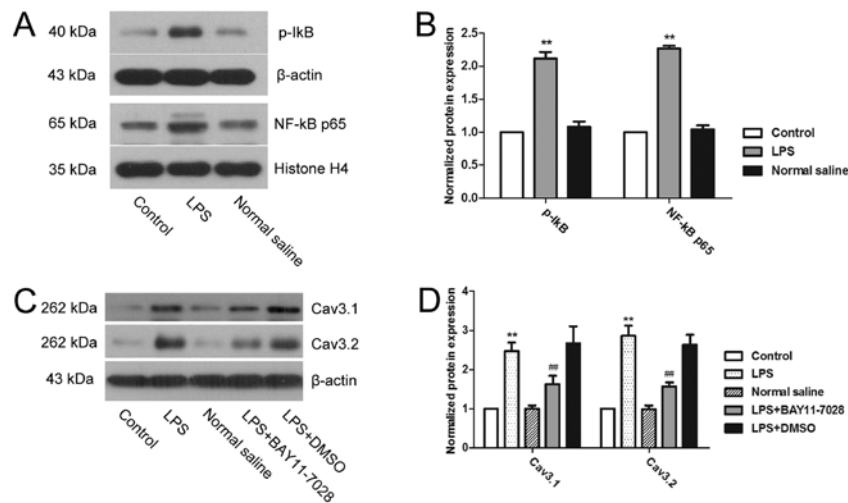


Figure 4. LPS increase Cav3.1 and Cav3.2 expression via the NF-κB signaling pathway. Western blot analysis was used to detect the expression of (A) p-IκB in the cytoplasm and NF-κB p65 in the nucleus; (B) β-actin and histone H4 were used as references to analyze the relative expression levels of p-IκB and NF-κB p65; respectively. (C) Western blotting detected the expression of Cav3.1 and Cav3.2; and (D) β-actin was used as an internal reference for the analysis of relative Cav3.1 and Cav3.2 expression levels. The western blot results in the figure are typical results from three repeated experiments, and the data are expressed as the means ± standard deviation. **P<0.01 compared to the control group; and ##P<0.01 compared to the LPS group. LPS, lipopolysaccharides; NF-κB, nuclear factor-κB.

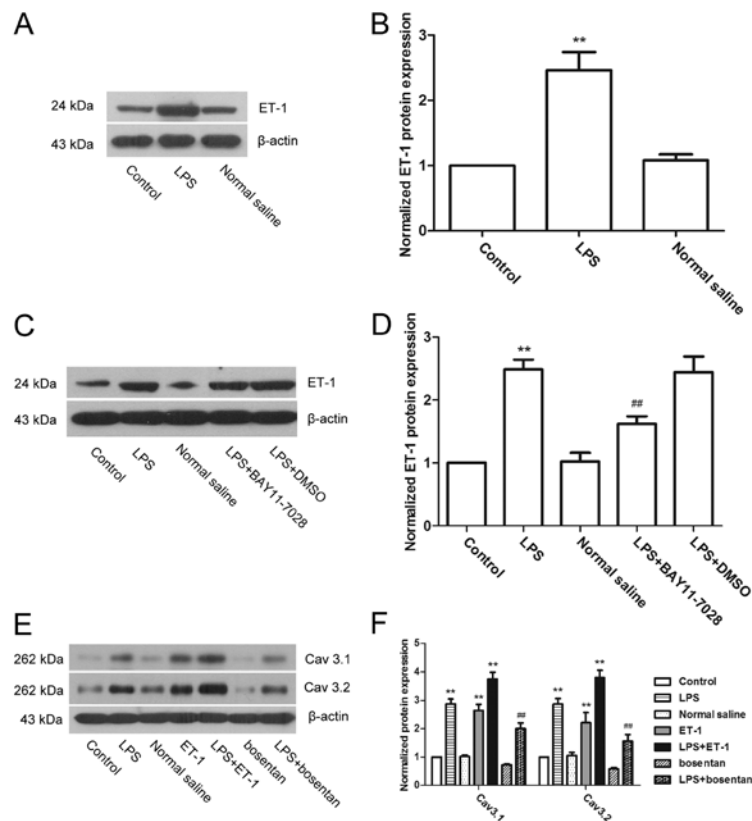


Figure 5. ET-1-mediated NF-κB signaling in LPS regulation of T-type calcium channels. Western blot analysis was used to detect the expression of (A, C) ET-1 and (E) Cav3.1 and Cav3.2; (B, D, F) β-actin was used as an internal reference for analysis of the relative expression level of each protein. The western blot results are typical results from three repeated experiments for each group. The data are expressed as the means ± standard deviation. **P<0.01 compared to the control group; and ##P<0.01 compared to the LPS group. ET-1, endothelin-1; NF-κB, nuclear factor-κB; LPS, lipopolysaccharides;

Cav3.1 and Cav3.2 and increased the concentration of calcium in the uterine smooth muscle cells. This process depends on the activation of the NF-κB/ET-1 signaling pathways. The above results show that T-type calcium channels play an important role in infection-induced calcium influx. T-type

calcium channels are expected to become a novel target for the prevention of preterm birth.

In the signal transduction process during uterine smooth muscle cell contraction in pregnant females, the ultimate step controlling contraction is the changes in cytosolic

calcium signaling (32). T-type calcium channels are a class of low activation potential, small single-channel conductance voltage-gated calcium channels that can directly regulate intracellular calcium ion concentrations. T-type calcium channels also play a role as a secondary messenger in a variety of physiological and pathological processes (23,35-37). T-type calcium channels expressed in uterine smooth muscle cells are mainly Cav3.1 and Cav3.2 subtypes (38). LPS is the main toxic component of Gram-negative bacteria (39). Levels of LPS were increased in the amniotic cavity of premature patients with bacterial infection (40). In the present study, the use of LPS for the treatment of uterine smooth muscle cells to mimic an *in vitro* bacterial infection microenvironment found that the expression of Cav3.1 and Cav3.2 was significantly upregulated in the infectious condition, and the intracellular calcium concentration also increased. However, T-type calcium channel antagonists can effectively inhibit the infection-induced increase of calcium concentration, indicating that T-type calcium channels play an indispensable role in the LPS signal transmission and the regulation of intracellular calcium concentration processes. Increased calcium concentration by excitable cells is known to lead to cell shrinkage (41). T-type calcium channels in the infectious condition are speculated to trigger uterine contractions, while T-type calcium channel antagonist agents can prevent preterm birth caused by infection.

NF- κ B is an important transcription factor. Activation can cause uterine smooth muscle cells to produce a variety of inflammatory cytokines and active substances, promoting intracellular calcium influx and inducing uterine contractions that lead to preterm birth (42). In the present study, under the treatment of LPS, there was a significant increase in the expression of cytosolic p-I κ B and NF- κ B p65 in the nucleus, suggesting that infection conditions can activate the NF- κ B signaling pathway, which has been confirmed in numerous previous studies (43,44). NF- κ B antagonists were further demonstrated to reverse the LPS-induced increase in Cav3.1 and Cav3.2 expression. Therefore, the increased expression of T-type calcium channels by infection partially depends on the activation of the NF- κ B signaling pathway.

ET-1 is one of the small proteins with strong vasoconstriction activities (45,46). Under stimulation, the uterus can produce ET-1 and release it in the paracrine form to induce contraction (47). The process of ET-1-induced uterine contractions ultimately depends on the increase in intracellular calcium concentration (48). In the present study, it was found that following LPS treatment the expression of ET-1 was elevated, whereas NF- κ B inhibitors reversed the effect of LPS on ET-1. Therefore, infection induces the release of ET-1 through the NF- κ B signaling pathway, which is consistent with previous studies (49,50). The present study also found that ET-1 can directly increase the expression of Cav3.1 and Cav3.2. In addition, ET-1 antagonists inhibited the LPS-induced upregulation of Cav3.1 and Cav3.2, indicating an important role of ET-1 in the upregulation of T-type calcium channels and calcium influx in uterine smooth muscle cells. Similar results have also been demonstrated in myocardial cells (51,52).

In conclusion, the results demonstrated that infection can induce the upregulation of T-type calcium channels and

facilitate calcium influx in uterine smooth muscle cells. This process is closely associated with the activation of the NF- κ B/ET-1 signaling pathway. The study illustrated the role of T-type calcium channels in premature birth caused by infection. T-type calcium channels are expected to become a novel target for the prevention of infection-induced preterm birth.

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