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Interaction between NF-κB and AP-1 and their intracellular localization at labor in human late pregnant myometrial cells in vivo and in vitro

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Abstract

Preterm birth (PTB) is the most important cause of neonatal morbidity and mortality next to congenital anomalies in the developed world. NF-κB and AP-1 were reported to play an important role in parturition initiation. However, the interaction relationship between the 2 molecules in labor initiation has not yet been reported.

This study aimed to investigate the interaction between NF-κB and AP-1 and their intracellular translocation during labor in human late pregnant myometrial cells (HLPMCs).

Co-immunoprecipitation (Co-IP), Western blot analysis, immunohistochemistry (IHC), and immunocytofluorescence (ICF) techniques were applied to explore the interaction between NF-κB and AP-1 and the alteration in their intracellular localization before and after labor onset.

The protein expression levels of NF-κBp65 and AP-1(c-jun) in the natural labor group were observed significantly higher than that in the non-labor group. Pearson's correlation analysis showed a positive correlation between the protein expression of NF-κBp65 and AP-1(c-jun). Interactions were found between the 2 molecules in HLPMCs both in natural labor and non-labor group and were also found in primary culture HLPMCs before and after neuromedin B (NMB) stimulation. NF-κBp65 and AP-1(c-jun) were localized mainly in the cytoplasm before labor onset or NMB stimulation and were translocated into the nucleus upon labor initiation and NMB stimulation.

These results demonstrated that upregulated protein expression of NF-κBp65 and AP-1(c-jun), the enhanced interaction between the 2 molecules, and their translocation to nucleus might be correlated to labor initiation.

Abbreviations: Co-IP = co-immunoprecipitation, CS = cesarean section, GPCR = G-protein-coupled receptor, HLPMCs = human late pregnant myometrial cells, ICF = immunocytofluorescence, IHC = immunohistochemistry, IOD = integrated optical density, NMB = neuromedin B, NMBR = neuromedin B receptor, PKC = protein kinase C, PTB = preterm birth, TFs = transcription factors, USMCs = uterine smooth muscle cells.

Keywords: AP-1(c-jun), cellular translocation, human late pregnant myometrial cells, interaction, labor, NF-κBp65

1. Introduction

Preterm birth (PTB), accounts for 11.1% of all deliveries worldwide, is the most important cause of neonatal morbidity and mortality not due to congenital anomalies in the developed world.[1] However, causes and mechanism of the labor onset and PTB are not yet clarified. The related studies worldwide, postulate that the inflammation plays a major role in the labor onset and PTB, and the inflammation theory becomes the most widely accepted hypothesis.[2–5] Although labor onset is a normal physiological process rather than an inflammatory response caused by exogenous pathogens, it is a process of inflammatory pathway activation and cascade amplification induced by endogenous factors.

Both NF-κB and AP-1 are the key transcription factors (TFs) in inflammatory response pathways. The expression of NF-κB in pregnant tissues and its regulating effect on downstream genes such as IL-1β, IL-6, IL-8, COX-2, TNF-α, and MMP-9 to induce labor have been validated by several basic research and clinical studies.[6,7] Previous studies of our group also demonstrated that activation of the RELA (NF-κBp65)/IL6-mediated pathway can induce labor onset in pregnant mice and influence the activity of myometrial primary cells in vitro.[8,9] AP-1, as another main TF in the regulation of inflammatory cytokines, is also receiving increasing attention for its potential role in labor onset.[10,11] It has been confirmed that NF-κB and AP-1could be activated to induce the expression of downstream inflammatory cytokines at the same time when stimulated by lipopolysaccharide (LPS).[12,13] Moreover, it has been reported that NF-κB and AP-1 were synchronously activated or suppressed in a protein kinase C (PKC) dependent manner to regulate expression of some pro-

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inflammatory genes.[14,15] The IL-8 promoter was also reported to contain binding sites for both NF-κB and AP-1,[16,17] and IL-1β promoted the expression of IL-8 gene through both NF-κB and AP-1 signaling pathways.[18] These studies indicated that NF-κB and AP-1 are both inflammatory TFs which could regulate expression of pro-inflammatory cytokines and the parturition initiation together. Therefore, we hypothesized that some interactions between NF-κB and AP-1 are most likely to exist in the process of parturition initiation, which were necessitating further studies.

Neuromedin B receptor (NMBR), which has been reported to be implicated in thermoregulation and stimulation of both urogenital and gastrointestinal smooth muscle contraction,[19] is a member of the G-protein-coupled receptor (GPCR) family and is a differentially expressed membrane receptor before and after labor onset revealed by the cDNA microarray chips in a preliminary study from our group.[20] Neuromedin B (NMB) is the specific agonist of NMBR. It has been reported that NMB binding to its receptor NMBR activates PKC- mitogen-activated protein kinase (MAPK) pathway,[19] and the NF-κB and AP-1 could be activated in a PKC-dependent manner. Our previous studies have demonstrated that NMB and its receptor can induce labor onset in pregnant mice and influence the activity of myometrial primary cells in vitro via a RELA (NF-κBp65)/IL6-mediated pathway.[8,9] Those findings strongly indicated that NMB binding to NMBR can activate both NF-κB and AP-1 pathways to induce labor initiation, and some interactions between NF-κB and AP-1 may exist to play a synergistic role in the process of parturition initiation. The present study proposed to use co-immunoprecipitation (CO-IP), western blotting, immunohistochemistry (IHC), and immunocytofluorescence (ICF) techniques to explore their interaction and intracellular localization changes in the myometrial cells in vivo and in vitro to discover the underlying mechanism of labor onset.

2. Materials and methods

2.1. Tissue collection

The study was approved by the Medical Ethics Committee of Xiangya Hospital Central South University (Approval ID: 201503428), and all participants provided written informed consent. The uterine smooth muscle biopsies from 12 term pregnant women of the lower uterine cesarean section (CS) incision and 6 women who underwent hysterectomy for cervical intraepithelial neoplasia (pathological examination prompted CINII-III, no carcinoma in situ and invasive cancer) were collected. The average age of the pregnant women was 29.3 ± 3.6 (25–34) years; average gestational age was 39.86 (39–40.43) weeks. The average age of the 6 women who underwent hysterectomy was 38 (35–40) years, and they consisted of the non-pregnancy group. The pregnancies were all singleton, not combined with medicochirurgical and obstetric complications. The CS was conducted because of pelvic stenosis, breech position, and protracted active phase. The pregnant women were divided into 2 groups based on their state of labor: the non-labor group (n = 6), and the natural labor group (n = 6). The non-labor group was defined as gestational age ≥39 weeks, no contractions detected by the electronic fetal monitor, no dilation of cervix, and the cervical Bishop score <4. The natural labor group was defined as gestational age ≥39 weeks, regular contractions detected by the electronic fetal monitor, dilation of cervix ≥6 cm, and underwent CS because of protracted active phase. No significant difference in the gestational age, neonatal weight, and Apgar scores of the pregnant women was observed between the 2 groups. Tissues were collected during the CS immediately after fetal delivery before oxytocin was injected into the uterine muscles. The tissues, excluding the endometrial and serous layer, were immediately frozen in liquid nitrogen and stored at –80°C until processed for protein extraction.

2.2. Primary culture of human late pregnant myometrial cells (HLPMCs)

HLPMCs were derived from the myometrial tissues on the edge of the lower uterine CS incision of the late-term pregnant women (n = 6) who were not in natural labor and whose inclusion criteria were the same as the non-labor group for tissue collection. The muscle tissues were immediately placed into the cold D-Hank's solution (Gibco) after biopsy and transported to the super-clean lab benchtop in 30 minutes. HLPMCs were cultured by the tissue explants adherent method as described previously.[21,22] The myometrial cells were passaged not more than 5 generations and divided into control group and NMB (10^{-10}M, 10^{-8}M, 10^{-6}M) groups.

2.3. Protein extraction and Western blot analysis

Total cellular proteins were extracted in the presence of protease inhibitors. The protein concentration was determined by a Bradford assay (Bio-Rad). Protein lysates (30 μg per sample) were separated by 10% SDS-PAGE and transferred to a polyvinylidenedifluoride membrane (PVDF; Millipore, Billerica, MA) at 300 mA, 4°C. The membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS) supplemented with 0.01% Tween 20 (TBST) for 2 hour and then incubated overnight at 4°C with primary antibodies at 1:1000 in TBST: Rabbit anti-p65 (# 8242; CST, UK), Rabbit anti-c-Jun (# 9165;CST, UK), and Rabbit anti-GAPDH (#2118; CST, UK). Subsequently, the membranes were washed 4 times (6 min each) with TBST and incubated with the appropriate secondary antibody for 1 to 2 hour at room temperature (1:2000; CST, UK) and then washed again with TBST. The detection of the immunoreactive bands was performed by enhanced chemiluminescence (ECL; GE Healthcare) using the Image-Quant LAS 4000 Imager (GE Healthcare), and densitometric analysis was conducted using Image-Quant TL (GE Healthcare).

2.4. Co-IP

Protein A agarose beads (Pierce Biotechnology, Rockford, IL) stored at 4°C were washed and diluted with protein lysate. Total cellular proteins were added to the pretreatment protein A agarose beads. The reactants were gently mixed at low speed at 4°C for 1 hour and briefly centrifuged at 14000 rpm. The protein mixtures were then incubated with 2 μL anti-p65 or anti-c-Jun antibodies at 4°C overnight, and rabbit IgG for negative control. Then Protein A agarose beads were added to capture the antigen-antibody complex, and the antigen-antibody mixture was shaken slowly at 4°C overnight. Instantly centrifuge at 14,000 rpm for 5 seconds and collect the agarose bead-antigen-antibody complex. Finally, the complex was separated on an SDS-PAGE gel, transferred to PVDF membrane, and immunoblotted with primary antibodies (anti-c-Jun or anti- p65), followed by secondary antibody, HRP labeled goat anti-rabbit.

2.5. IHC

Paraffin-embedded tissue blocks were sliced into 3–4 μm sections. IHC was performed using a Ventana XT automated stainer (Ventana Corp., Tucson, AZ). Sections were deparaffinized using EZ Prep solution (Ventana). CC1 standard (pH8.4 buffer containing Tris/borate/EDTA) was used for antigen retrieval and blocked with inhibitor D (3% H_2O_2) for 4 minutes at 37°C. The slides were incubated with primary antibodies, p65 or c-Jun at 1:100 for 40 minutes at 37°C followed by a universal secondary antibody for 20 minutes at 37°C. The slides were incubated with streptavidin-horseradish peroxidase (SA-HRP) avidin D for 16 minutes at 37°C and then the substrate, 3,3′-diaminobenzidine tetrahydrochloride (DAB) H_2O_2, was added for 8 minutes followed by hematoxylin and eosin (HE) counterstaining at 37°C. The positive expression of p65 and c-Jun was defined as brownish yellow granules appearing in the cytoplasm and nucleus. Five high power field images were randomly selected from each slide to measure the integrated optical density (IOD) values and calculate the average value using the Image-Pro Plus 6.0 software (Media Cybernetics).

2.6. ICF

Cell slides were fixed with 4% paraformaldehyde in PBS pH7.4, subjected to antigen retrieval by incubation in sodium citrate buffer pH6.0 at 105°C for 20 min, and permeabilized with 0.3% Triton X-100. The slides were then subjected to 5% BSA blocking and incubation with primary antibodies (p65 1:200, c-Jun 1:100) overnight at 4°C. Subsequently, the slides were incubated with the fluorescent labeled goat anti-rabbit secondary antibody for 1 hour and stained with 4,6-diamidino-2-phenylindole (DAPI; Genview) for 5 minutes. Finally, the antifade mounting medium was added to the slides and sealed with coverslips, respectively. The images were captured by fluorescence microscopy. The positive expression of p65 was defined as green fluorescence in the cytoplasm and nucleus and c-Jun as red. Ten high power field images were randomly selected on each slide to measure the IOD values and calculate the average using the Image-Pro Plus 6.0 software (Media Cybernetics).

2.7. Statistics analyses

Results are presented as mean ± SD. We used 1-way analysis of variance followed by Tukey's HSD post hoc test. Pearson analysis was performed for analysis of the relationship between protein expression of NF-κB and AP-1. A P value less than 0.05 was considered significant.

3. Results

3.1. Identification of HLPMCs in vivo and in vitro and verification of the expression of NMBR in the uterine smooth muscle cells (USMCs)

The biopsy tissues from the edge of the lower uterine CS incision were confirmed as uterine smooth muscles by HE staining (Fig. 1A). alpha smooth muscle-actin (α-SMA) and NMBR expression were detected by IHC (Fig. 1B,1C). Primary culture of HLPMCs primarily displayed long fusiform shape and a small part of polygon shape under the inverted microscope. α-SMA and NMBR expressions were also detected by immunocytochemistry (Fig. 1E, 1F).

3.2. Expression level and intracellular localization changes of p65 and c-Jun in HLPMCs before and after labor onset

Western blot analysis showed that average protein expression levels of p65 and c-Jun in the natural labor group were significantly higher than that in the non-labor and non-pregnancy group ($P < .05$, Fig. 2B). IHC analysis showed that protein expression levels of p65 and c-Jun in the nucleus of the natural labor group were significantly higher than that in the non-labor

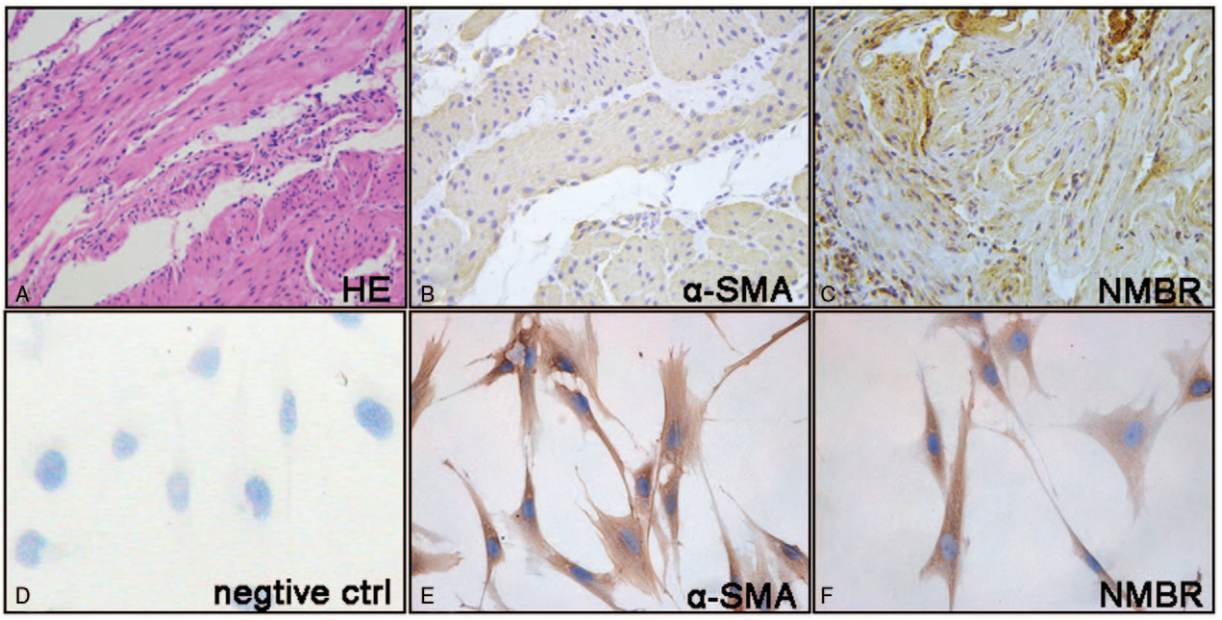

Figure 1. Primary culture and identification of HLPMCs. A. HE staining of human late pregnant myometrial tissues (HLPMTs). B. α-SMA expressed positively in HLPMTs. C. NMBR expressed positively in HLPMTs. D. Negative control. E. α-SMA expressed positively in primary culture myometrial cells. F. NMBR expressed positively mainly in the membranes of primary culture myometrial cells. α-SMA = alpha smooth muscle-actin, HE = hematoxylin and eosin, HLPMCs = human late pregnant myometrial cells, HLPMTs = human late pregnant myometrial tissues, NMBR = neuromedin B receptor.

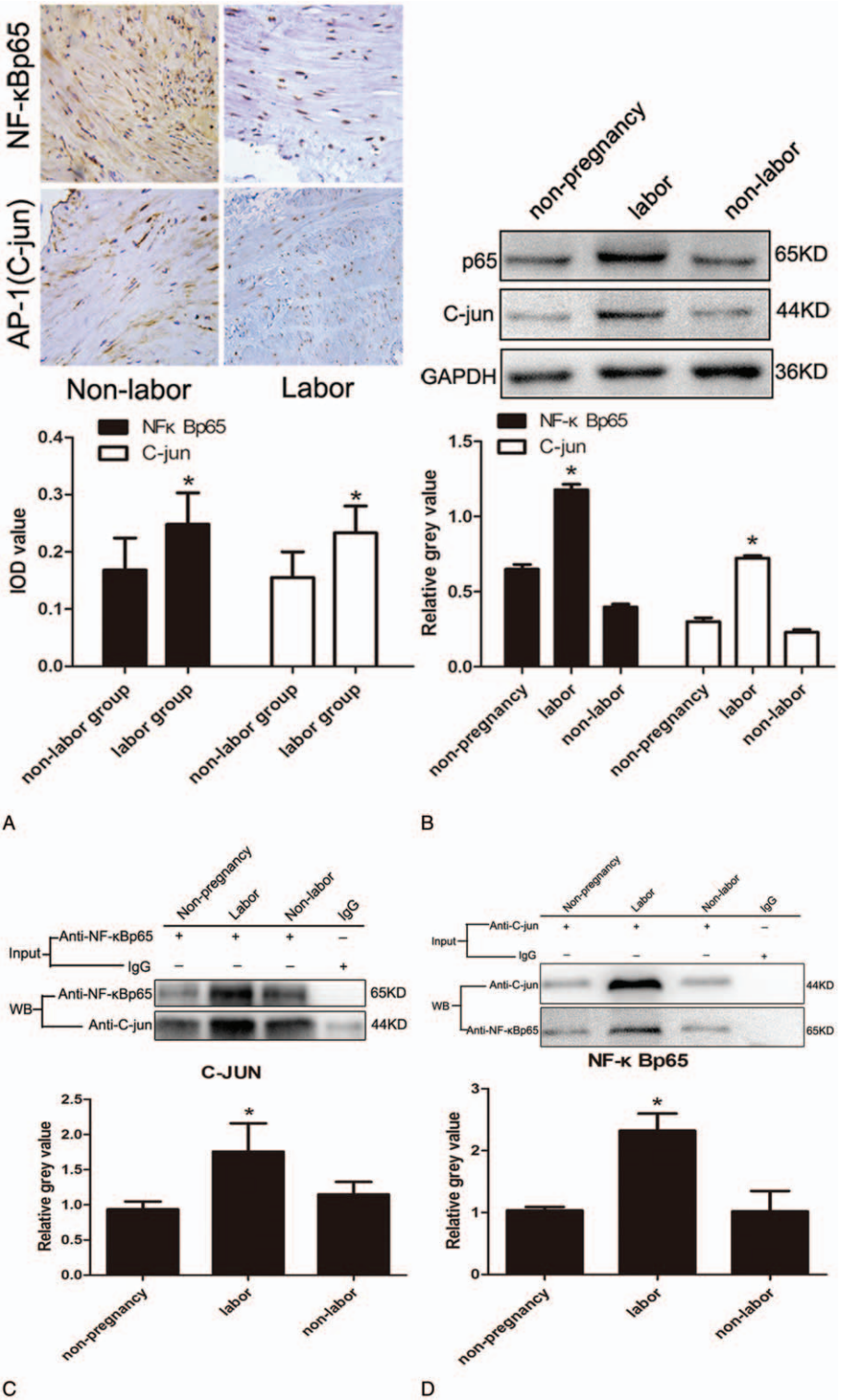

Figure 2. Expression level, intracellular localization changes and Interaction of p65 and c-Jun in HLPMCs before and after labor onset. A. In non-labor group, dispersed brownish yellow granules were detected in the cytoplasm, while in labor group, concentrated brownish yellow granules were detected in the nucleus, indicating that p65 and c-Jun mainly expressed in the cytoplasm before labor onset and translocated to the nucleus after labor onset. Semi-quantitative analysis by IHC showed that p65 and c-Jun protein expression level in the nucleus was higher in the natural labor group than that in the non-labor group ($P < .05$). B. Western blot analysis showed that protein expression of p65 and c-Jun in labor group was higher than that of the non-labor and non-pregnancy groups ($P < .05$). C. Co-IP analysis showed that c-Jun protein existed in the protein complexes pulled down by anti-p65 anti-body before and after labor onset, and protein expression of c-jun was the highest in the labor group ($P < .05$). D. Co-IP analysis showed that p65 protein existed in the protein complexes pulled down by anti-c-Jun anti-body before and after labor onset, and protein expression of p65 was the highest in the labor group ($P < .05$). Co-IP = co-immunoprecipitation, HLPMCs = human late pregnant myometrial cells, IHC = immunohistochemistry

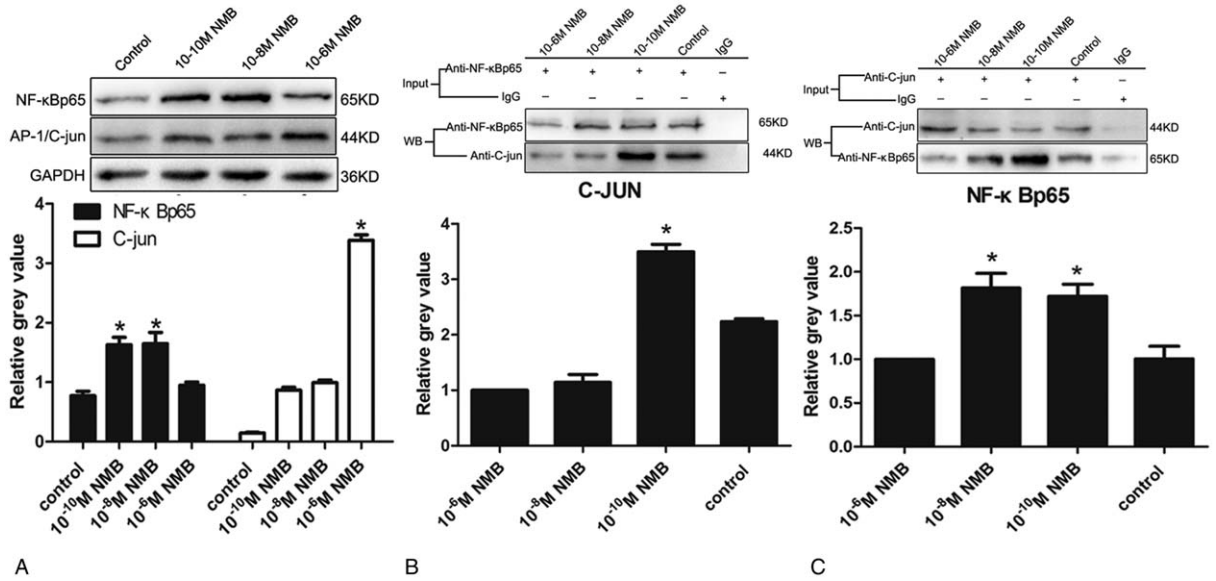

Figure 3. Expression level and Interaction of p65 and c-Jun in HLPMCs stimulated by NMB. A. Western blot analysis showed that protein expression of p65 and c-Jun was significantly upregulated after NMB stimulation in 10^{-10}–10^{-8}M concentration range ($P < .05$), however, in the concentration of 10^{-6}M, protein expression of p65 decreased with no significant difference when compared with the control group ($P > .05$ versus control group) but c-Jun protein expressed the highest ($P < .05$). B. Co-IP analysis showed that c-Jun protein existed in the protein complexes pulled down by anti-p65 anti-body before and after NMB stimulation, and protein expression of c-jun was the highest in the concentration of 10^{-10}M ($P < .05$). C. Co-IP analysis showed that p65 protein existed in the protein complexes pulled down by anti-c-jun anti-body before and after NMB stimulation, and protein expression of p65 was the highest in the concentration of 10^{-10} to 10^{-8}M ($P < .05$). Co-IP = co-immunoprecipitation, HLPMCs = human late pregnant myometrial cells, NMB = neuromedin B.

group ($P < .05$, Fig. 2A). Interestingly, IHC analysis also showed that before labor onset, both proteins were mainly localized in the cytoplasm and partially in the nucleus. After labor onset, however, almost all of both proteins were located in the nucleus and could barely be seen in the cytoplasm (Fig. 2A). This indicated that p65 and c-Jun were upregulated expressed and translocated from cytoplasm to the nucleus during parturition initiation.

3.3. Interaction between p65 and AP-1 in HLPMCs at labor

Pearson's correlation analysis showed that the correlation coefficient of protein expression levels of p65 and c-Jun of IHC analysis in the natural labor group and non-labor group were 0.659 ($P = .038$) and 0.879 ($P = .001$), respectively, which indicated that expression level of p65 had a highly positive correlation with that of c-Jun in both of the groups. Co-IP and Western blot analysis showed that in HLPMCs c-Jun was present in the protein complexes pulled down by p65 antibody (Fig. 2C), and p65 was detected in the protein complexes pulled down by the c-Jun antibody (Fig. 2D). And protein expression of c-jun and p65 were detected the highest in the labor group ($P < .05$, Fig. 2C, 2D). These indicated that a potential interaction of p65 and AP-1 existed in HLPMCs and was enhanced at labor.

3.4. Expression level and intracellular localization changes of p65 and c-Jun in HLPMCs in vitro before and after NMB stimulation

Western blot analysis in vitro indicated that the expression level of p65 and c-Jun was significantly upregulated after NMB stimulation in 10^{-10}–10^{-8}M concentration range ($P < .05$, Fig. 3A). ICF analysis showed that p65 and c-Jun translocated from the cytoplasm to the nucleus consecutively and their expression localization almost overlapped in the primary culture HLPMCs after NMB stimulation in the concentration of 10^{-10}M (Fig. 4). Semi-quantitative analysis demonstrated that the expression IOD value of p65 and c-Jun in the NMB group was significantly greater than that in the control group ($P < .05$). This indicated that the NMB stimulation could elevate p65 and c-Jun protein expression levels and promote their translocation to the nucleus.

3.5. Interaction between p65 and AP-1 in HLPMCs by NMB stimulation

Co-IP and Western blot analysis also showed in vitro that c-Jun was present in the protein complexes pulled down by p65 antibody (Fig. 3B), which was detected the highest in the concentration of 10^{-10}M ($P < .05$, Fig. 3B). p65 was detected in the protein complexes pulled down by the c-Jun antibody (Fig. 3C), which was detected the highest in the concentration of 10^{-10}–10^{-8}M ($P < .05$, Fig. 3C). Pearson's correlation analysis of ICF showed that the expression level of p65 was positively correlated with the expression level of c-Jun after stimulation with NMB ($r = 0.874$, $P < .05$). Those results indicated that a potential interaction of p65 and AP-1 existed in HLPMCs in vitro and the interaction expressed strongest in the concentration of 10^{-10}M of NMB.

4. Discussion

Regular uterine contraction and cervical dilation are regarded as indicators of parturition. USMCs were the final effectors of parturition. Thus, the differentially expressed products between natural labor and non-labor USMCs may be closely related to labor onset. Primary cell cultures have been shown to retain morphological and functional characteristics and expression of NMBR were shown to be retained for at least 4 generations.[9]

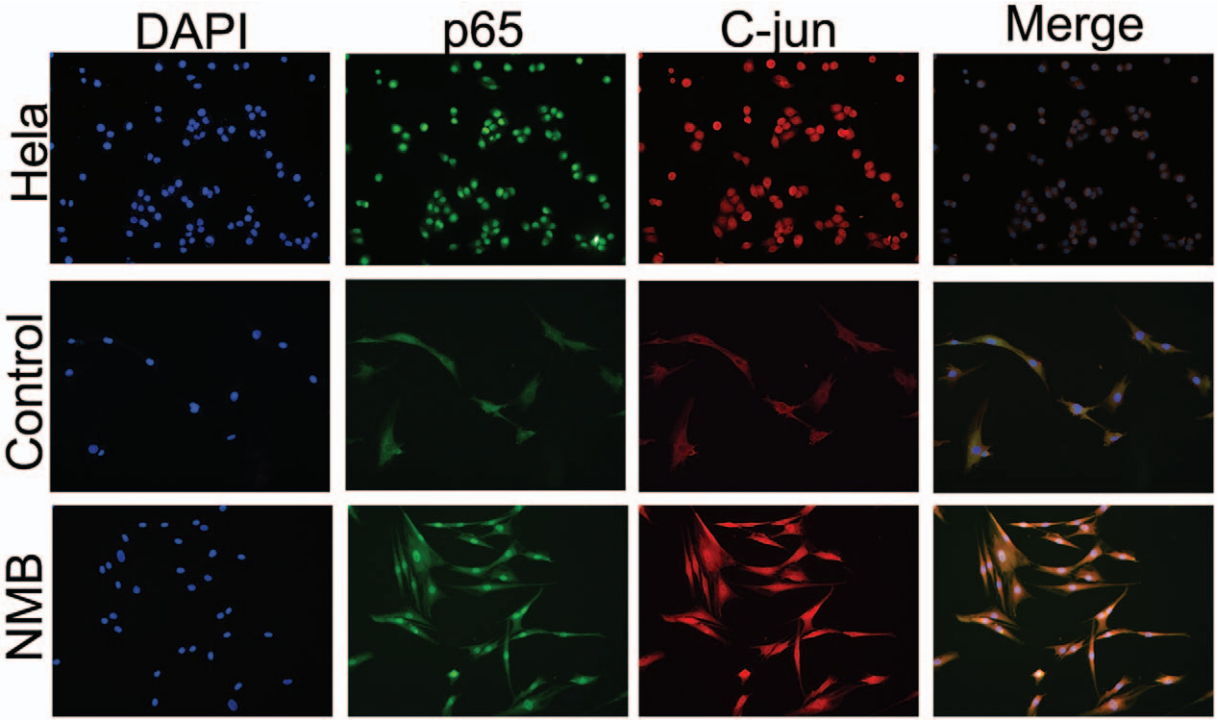

Figure 4. ICF images of p65 and c-Jun before and after NMB stimulation. DAPI is the specific nuclear chromogenic agent with blue fluorescence. HeLa cells that confirmed the protein expression of both p65 and c-Jun were used as the positive control. The green and red fluorescence showed p65 and c-Jun expression, respectively. The images showed that in contol group, p65 (green fluorescent) and c-Jun (red fluorescent) mainly expressed in the cytoplasm, while in 10^{-10}M NMB stimulation group, p65 (green fluorescent) and c-Jun (red fluorescent) mainly expressed in the nucleus. DAPI = 4,6-diamidino-2-phenylindole ,ICF = immunocytofluorescence, NMB = neuromedin B.

This study explored the interactions between NF-κB and AP-1 and their intracellular localization changes the first time in the USMCs to discover the underlying mechanism of labor onset, by using HLPMCs before or after labor onset in vivo and primary culture HLPMCs in vitro. Standard immunochemical staining techniques were used to demonstrate positive expression of a-SMA and NMBR in the primary culture of myometrial cells, thus confirming the myogenic origin of cultured cells.

NMBR is a member of the GPCR family and was confirmed a differentially expressed membrane receptor before and after labor onset in HLPMCs in a preliminary study from our group.[20] Our previous study also confirmed that NMB, the specific agonist of NMBR, and its receptor can induce the labor onset in pregnant mice and influence the activity of primary myometrial cells in vitro via a RELA (NF-κBp65)/IL6-mediated pathway.[8,9] Therefore, the present study used NMB to stimulate primary culture HLPMCs in vitro to verify the interaction of NF-κB and AP-1 and their intracellular localization changes.

NF-κB and AP-1 are 2 key TFs that have played major roles in the process of endogenous inflammation pathway activation and the subsequent cascade amplification process. Their effects in labor onset have attracted attention worldwide. As a critical nuclear TF, NF-κB played a vital role both in the physiological and pathological processes of delivery in USMCs.[23] p65 is the most common and the primary functional unit of NF-κB. Preliminary study from our group and several other studies have confirmed that NF-κBp65 plays a key role both in term and preterm labor onset through the regulation of expression of the oxytocin receptor, pro-inflammatory cytokines, and signal transduction molecules.[8,9,24,25] As another key nuclear TF in the inflammatory pathways, AP-1 is mainly involved in regulating the expression of pro-inflammatory cytokines and the differentiation or metastasis of tumor cells. The primary functional unit of AP-1 is c-Jun. As an important TF, its role in the process of labor onset has also been closely monitored.[10,11,26]

Protein expression of p65 and c-Jun in this study were both verified higher in the natural labor group than in the non-labor group by Western blotting and IHC methods, which demonstrated that p65 and c-Jun might play important roles in parturition at the same time. Further analysis showed that expression levels of p65 and c-Jun were highly correlated in the same labor condition, especially in the natural labor group ($r = 0.879$, $P = .001$). p65 and c-Jun were primarily localized in the cytoplasm before labor onset but translocated to the nucleus upon labor initiation. The results indicated that the expression level of p65 and c-Jun was up-regulated with a positive correlation and had a synchronous localization change when the state of the USMCs altered from resting to contraction. Thus, we can confirm that it can induce synchronous changes in both protein expression level and intracellular translocation in p65 and c-Jun upon labor onset. The results confirmed that NF-κB and AP-1 played a synergistic role in labor onset and the occurrence of an interaction correlation may lead to the co-translocation to the nucleus. Therefore, we further investigated the interaction between p65 and c-Jun and its role in intracellular translocation by using Co-IP, Western blot, and ICF analyses.

As key TFs of inflammation pathways, NF-κB and AP-1 have been widely studied in multiple fields.[27–29] However, the interaction relationship between them has not yet been reported. Co-IP and Western blot analyses of this study preliminarily confirmed that some interactions indeed occurred between NF-κB and AP-1 in HLPMCs and the interaction enhanced upon labor onset and NMB stimulation. Furthermore, IHC and ICF analyses confirmed that NF-κB and AP-1 localized in the

cytoplasm before labor onset and NMB stimulation, whereas translocated to the nucleus upon labor initiation and stimulated by NMB. Semi-quantitative analysis showed that the expression level of NF-κB and AP-1 elevated after labor onset and NMB stimulation. This indicated that NF-κB and AP-1 expression elevation and co-translocation to the nucleus might the potential mechanism inducing labor onset. NF-κB and AP-1may be activated and translocated to the nucleus to bind to the DNA binding site of the promoters in order to upregulate the expression of downstream related genes such as IL-6, COX-2, and MMP9, to induce labor onset. Moreover, NF-κB and AP-1 may combine with each other directly or indirectly when they are co-translocated to the nucleus.

In conclusion, NF-κB and AP-1 played a synergistic role in labor onset and their protein expression levels elevated and co-translocated to the nucleus upon labor initiation and NMB stimulation. Some interactions between the 2 proteins exist in the whole process, which plays an important role in NF-κB/AP-1-induced labor onset pathways. However, the Co-IP combined with Western blot analysis used in this study to verify the interaction between NF-κB and AP-1 can only demonstrate that NF-κB and AP-1 both existed in the pulled-down complexes, indicating that the interactions between them could be direct or indirect, or with a possibility of interactions mediated by other proteins. Thus, further approaches such as chromatin immunoprecipitation assay (ChIP), GST pull-down, point mutation, and siRNA should be employed to study the interaction locus, interaction style, and the mechanism of interaction between NF-κB and AP-1 in the process of labor initiation.

Author contributions

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Validation: Weishe Zhang.
Visualization: Weishe Zhang.
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