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Source: Biology of Reproduction, 86(5)

Published By: Society for the Study of Reproduction

URL: <https://doi.org/10.1095/biolreprod.111.095984>

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Neuromedin B and Its Receptor Influence the Activity of Myometrial Primary Cells In Vitro Through Regulation of *Il6* Expression via the *Rela/p65* Pathway in Mice¹

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ABSTRACT

The neuromedin B receptor (*Nmbr*) is an important physiological regulator of spontaneous activities and stress responses through different cascades as well as its autocrine and paracrine effects. Previous studies have revealed that neuromedin B (*Nmb*) and its receptor signal via the *Rela* (also known as *p65*)/*Il6* pathway in a mouse model of pregnancy. This study investigated the mechanism of *Nmbr* signaling via the *Rela/p65-Il6* pathway and regulation of the concentration of intracellular free calcium ($[Ca^{2+}]_i$) during the onset of labor in primary mouse myometrial cell cultures isolated from mice in term labor. Data demonstrated *Nmbr* agonist-mediated upregulation of the DNA binding activity of *Rela/p65*, *Il6* expression, and $[Ca^{2+}]_i$ in a concentration-dependent manner. Furthermore, a significant correlation was observed between DNA binding activity of *Rela/p65* and *Il6* expression. Moreover, this up-regulation was blocked by *Nmbr* and *Rela/p65* knockdown, achieved by RNA interference (RNAi) technology. No significant differences were identified in the inhibition of *Il6* expression as a result of *Nmbr* or *Rela/p65* knockdown. However, significant differences were observed between the $[Ca^{2+}]_i$ in *Rela/p65*-specific group and that in the *Nmbr*-specific small interfering RNA (siRNA)-treated groups. These data demonstrated that the *Nmb/Nmbr* interaction in pregnant myometrial primary cells in vitro predominantly influenced uterine activity through regulation of *Il6* expression via the *Rela/p65* pathway, although the effects of *Nmbr* on $[Ca^{2+}]_i$ involved several pathways that remain to be elucidated.

Il6, myometrium, neuromedin B, neuromedin B receptor, neuropeptides, null mutation/knockout, parturition, *Rela/p65*, transcriptional regulation

INTRODUCTION

Preterm birth is a leading cause of neonatal mortality and a major cause of pediatric morbidity and long-term disability. The global rate of preterm delivery is increasing and there is no effective means of prevention [1]. Current strategies to prolong pregnancy are based on inhibition of uterine myometrium contraction in preterm labor [2–4]. However, the mechanism

by which spontaneous onset labor is initiated, both at term and preterm, is still unclear. Therefore, the effects of the tocolysis in preventing preterm labor do not satisfy clinical needs [5, 6]. Other strategies have been designed to maintain a state of uterine quiescence and pregnancy, preventing the uterine smooth muscle from initiating contractions and entering preterm labor [7, 8]. The use of cDNA microarray technology is critical for the identification of novel targets related to the contraction of uterine smooth muscles, in order to provide a greater understanding of the mechanisms underlying preterm birth. It has been reported that the neuromedin B receptor (*Nmbr*) gene is one of the group of G protein-coupled receptors (GPCR), which are typical drug targets that are differentially expressed during parturition, while oxytocin receptor genes and other tocolytic targets do not exhibit such changes in expression [9, 10]. Furthermore, the expression of *Nmbr* peaks at term and occurs before parturition in humans and mice, and the *Nmbr* agonist neuromedin B (NMB) shortens the gestational age of mice with an associated increase in DNA binding activity of *Rela/p65* and *Il6* mRNA expression [11]. These results suggest that the mechanism underlying the onset of labor involves changes in the expression of the *Nmbr*, *Rela/p65*, and *Il6* genes in a mouse model of pregnancy. Other studies have shown that NMB is a member of the family of bombesin-like peptides that, like oxytocin, are predominantly secreted by the hypothalamus and pituitary gland [12]. NMB binds with high affinity to the NMBR to mediate the biological effects of smooth muscle contraction, spontaneous activity, and stress responses [13, 14]. These studies implicate *Nmbr* as a novel tocolytic drug target. Furthermore, the biological effects of the *Nmb/Nmbr* interaction are mediated through the *Gpcr* pathway, resulting in phospholipase C/protein kinase C (PKC)-mediated myosin light chain phosphorylation and increased intracellular free calcium ($[Ca^{2+}]_i$) [12–14], which induce uterine smooth muscle cell contraction. Related research has demonstrated that PKC activates *Rela/p65* [15–17], and there is now compelling evidence demonstrating that *Rela/p65* is an important upstream regulator of labor-associated processes [18]. Indeed, *Rela/p65* and its associated inflammatory reactions are widely accepted to be a key feature of labor onset [19–21]. Downstream effectors in the *Rela/p65* pathway, including *Il6* and interleukin 1b (*Il1b*), have been shown to promote cervical ripening, uterine contraction, and acceleration of human parturition [22–24]. Therefore, it is hypothesized that *Nmb/Nmbr* regulates uterine smooth muscle contraction in parturition through regulation of the expression of and $[Ca^{2+}]_i$ via the *P65* pathway.

This study evaluated the role and mechanism of the *Nmb/Nmbr* interaction in regulation of the DNA binding activity of *Rela/p65*, *Il6* expression, and $[Ca^{2+}]_i$ in primary murine myometrium cells isolated at the onset of labor. Furthermore, regulation of *Il6* expression and $[Ca^{2+}]_i$ was investigated by

¹Supported by the National Natural Science Foundation of China (30672241) and the Postdoctoral Science Foundation of China (20070410312).

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Received: 29 August 2011.

First decision: 25 September 2011.

Accepted: 27 December 2011.

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eISSN: 1529-7268 <http://www.biolreprod.org>

ISSN: 0006-3363

small interfering RNA (siRNA)-mediated knockdown of *Nmbr* and *Rela/p65*.

MATERIALS AND METHODS

Primary Culture of Myometrial Cells and Identification

All animals received humane care in compliance with the university's guidelines. Experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Central South University. Inbred BALB/c pregnant mice were killed after parturition of the first neonate. Uterine horns and cervix were immediately excised, and fetoplacental units were removed. Myometrial tissue was rapidly isolated from connective tissue and adherent endometrium by scraping, under the guidance of histology in pre-experiment, and cut into fragments (approximately 1 mm³). Myometrial tissue was digested using 375 U/ml collagenase type II (Sigma) containing 25 U/ml phosphate saline at 37°C in 95% humidified atmosphere containing 5% CO₂ with agitation. Dissociated myometrial cells were collected by centrifugation (500 × g for 15 min), and resuspended in sterile, phenol red-free Dulbecco modified Eagle medium (DMEM; Gibco, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS), 25 mM HEPES, 100 U/ml penicillin-streptomycin. Medium was replaced by fresh DMEM supplemented with 20% FCS 1 day later and by 10% FCS on the following day. Cells were used at confluence 3 days after plating. Myometrial cells were plated on 6-well silicone elastomer Flex I culture plates coated with type I collagen (Flexcell International Corp., McKeesport, PA) at a density of 3 × 10⁶ cells per well. The purity of the uterine smooth muscle cells (SMCs), and the expression of NMBR in the primary cultured cells was confirmed as described below.

Experimental Protocols

Myometrial cells were cultured for 72 h in 10% FCS/DMEM before being randomly divided into experimental groups and incubated for 24 h in 10% FCS-DMEM. The control groups were divided into untreated cells (control group); Lipofectamine 2000-treated (transfection control group); and no-sense siRNA-treated (no-sense control group). Four NMB-treated groups were prepared, consisting of NMB dosed at 10⁻¹⁰ M, 10⁻⁸ M, 10⁻⁶ M, and 10⁻⁴ M (10⁻¹⁰ M ~ 10⁻⁴ M), respectively. Four parallel cultures were prepared to assess the effects of siRNA-mediated knockout of *Nmbr* (*Nmbr* siRNA+NMB) as follows: NMB (10⁻¹⁰ M ~ 10⁻⁴ M) after 4 h pretreatment with *Nmbr* siRNA. An additional four parallel cultures were prepared to assess the effects of siRNA-mediated knockout of *Rela/p65* (*p65* siRNA+ NMB): NMB (10⁻¹⁰ M ~ 10⁻⁴ M) after 4-h pretreatment with *Rela/p65* siRNA. Six replicates of each experimental culture were prepared.

siRNA Preparation and Selection

Knockdown of *Nmbr* (GenBank accession number NM-008703) and *Rela* gene (GenBank accession number NM-009045, also known as *p65*) was achieved by the transfection of sequence-specific siRNA. The dominant *Nmbr* and *Rela/p65* sequences and negative control sequences were constructed by Genepharma Company (Shanghai, China). Blast analysis was performed to confirm target gene specificity of the designed siRNA duplexes. Transfections were carried out at a final concentration of 100 nM, using Lipofectamine2000 (Invitrogen), according to the manufacturer's instructions. Medium was changed 24 h after transfection, and analyses were performed after a further 24 h. Transfection efficiencies were validated by visualization of coexpressed enhanced green fluorescent protein under fluorescence microscopy. The effects on cell growth were measured by methyl thiazolyl tetrazolium (MTT) assay according to the reference methods [25]. The knockdown efficiency of three independent siRNA pairs were analyzed (their sequences were shown in Annex 1) by investigation of reductions in target gene specific mRNA and protein levels using real-time PCR and immunocytochemistry (ICC), data not shown. The most effective siRNA were confirmed as *Nmbr* -987 siRNA and *Rela/p65*-1477 siRNA (Table 1).

Rela/p65 DNA Binding Activity Assay

Nuclear proteins were extracted from myometrial cells, and relative *Rela/p65* DNA binding activity was quantified and calculated using the NoShift transcription factor assay kit (Merck Corporation) according to the manufacturer's instructions.

RNA Extraction and Real-Time PCR

Real-time PCR analyses of *Nmbr*, *Rela/p65*, and *Il6* mRNA expression were performed as previously described [25]; primer sequences are showed in

TABLE 1. siRNA targeting *Nmbr* and *Rela/p65* gene sequences.

siRNA	Direction	Sequence
<i>Nmbr</i> -420	Sense	5'-GGUACAGAGCUAUCGUGAATT-3'
	Antisense	5'-UUCACGUAAGCUCUGUACCTG-3'
<i>Nmbr</i> -589	Sense	5'-CAAAGAAUGUGGUGACCAATT-3'
	Antisense	5'-UUGGUGACCAUUCUUUGTA-3'
<i>Nmbr</i> -987*	Sense	5'-GCUUAGGAAGCACUUAATT-3'
	Antisense	5'-UUGAAGUCUCCUAAAGCTT-3'
<i>Rela/p65</i> -216	Sense	5'-GAAUCUCCUGGUCACCAATT-3'
	Antisense	5'-UUGGUGACCAUUCUUUGTA-3'
<i>Rela/p65</i> -909	Sense	5'-GGACCUAUGAGACCUUAATT-3'
	Antisense	5'-UUGAAGUCUCAUAGGUCCTT-3'
<i>Rela/p65</i> -1477*	Sense	5'-GCUUAGGAAGCACUUAATT-3'
	Antisense	5'-UUGAAGUCUCCUAAAGCTT-3'
Negative control sequence	Sense	5'-UUCUCCGAACGUGUCACGUTT-3'
	Antisense	5'-ACGUGACACGUUCGGAGAATT-3'

* The most effective siRNA are indicated in boldface. The number is the location of the mRNA sequence on the target gene which is blocked via siRNA interference.

Table 2. Briefly, RNA was extracted from SMCs using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA samples were first treated with DNase I (Qiagen) according to the manufacturer's instructions to remove possible DNA contamination. mRNA was reverse transcribed to cDNA with ReverTra Ace (TOYOBO). Quantitative analysis of gene expression was performed with the ABI Prism 7500 sequence detection system (PE Applied Biosystems) using SYBR Green real-time PCR Master Mix-Plus (TOYOBO). Values for target genes were related to their controls using the 2^{-ΔΔCt} calculation method [26]. Absolute gene transcription was normalized to the reaction with an end volume of 25 μl. Primers used for the analysis of gene transcription are described in Table 2. The PCR amplification conditions were as follows: 95°C for 5 min, 94°C for 20 sec, 55°C for 20 sec, 72°C for 20 sec, 72°C for 5 min, and 55°C for 10 sec for 30–35 cycles. Total RNA (1 μg) was then reverse transcribed using the Superscript III reverse transcription kit (Invitrogen) according to the manufacturer's instructions.

Detection of Protein Expression

Protein expression of α-smooth muscle actin (α-SMA) was analyzed by direct immunofluorescence and ICC using fluorescein isothiocyanate (FITC)-labeled α-SMA antibody (Sigma-Aldrich) and the primary antibody α-SMA (1:200 dilution; Santa Cruz Biotechnology), respectively. Protein expression of *Nmbr* and *Rela/p65* was detected by ICC using the primary detection antibodies to *Nmbr* (code sc-34376) and *Rela/p65* (1:200 dilution; Santa Cruz Biotechnology). Interleukin 6 (IL6) protein in culture supernatants was detected by ELISA using mouse IL6 Immunoassay Kits (Biosource) according to the manufacturer's instructions.

Measurement of Intracellular Free Calcium Concentration

Cells were seeded on culture slides and washed with serum-free RPMI 1640 medium twice before loading with 1% fluo-3 AM (code sc-202612; Santa Cruz Biotechnology) fluorescent indicator dye at 37°C for 45min in the absence or presence of CaCl₂ 2 mM (pH 7.4). Slides were washed three times with distilled water to remove extracellular fluo-3 AM. Calcium concentration [Ca²⁺]_i was determined using laser confocal scanning microscopy (model TCS-SP5; Leica) according to the manufacturer's instructions.

TABLE 2. Primers used for *Nmbr*, *Rela/p65*, and *Il6*.

Primer	Direction	Sequence	Size (bp)
<i>Nmbr</i>	Sense	5'-CATGCGGAATGTCCCTAACATC-3'	331
	Antisense	5'-CCAAGCTACCAATGCGTGCTAC-3'	
<i>Rela/p65</i>	Sense	5'-GCAGAAAGAACATTGAGG-3'	225
	Antisense	5'-TCATCTGTGTCTGGCAAGTAA-3'	
<i>Il6</i>	Sense	5'-CCACGGCCTTCCCTACTTC-3'	499
	Antisense	5'-TTGGTCCTTAGCCTCCT-3'	
<i>β-actin</i>	Sense	5'-AATGGGTGAGGAGGACTCCT-3'	250
	Antisense	5'-ACGGTTGGCCTTAGGTTTCAG-3'	

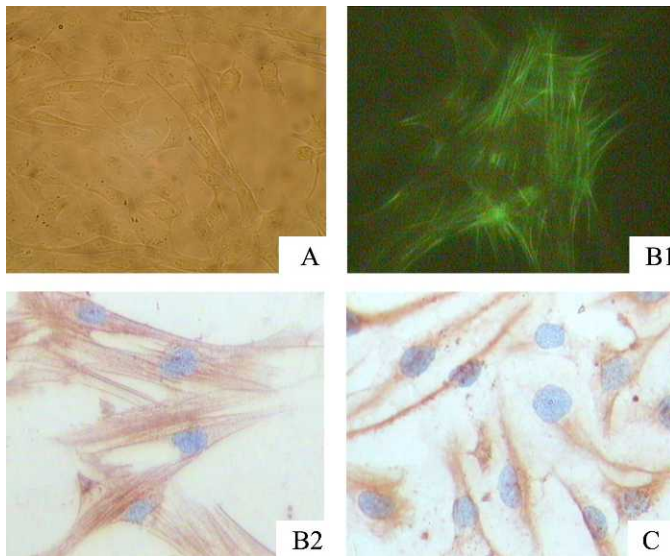


FIG. 1. The cultured primary SMCs from term myometrium and verification. **A)** Cultured primary SMCs within passage 4 were observed to be long and fusiform in shape by inverted microscopy. **B1** and **B2)** The positive expression of α -SMA by direct immunofluorescence (**B1**) and immunocytochemistry (**B2**). **C)** The positive expression of NMBR by immunocytochemistry. Original magnification $\times 100$ (**A**) and $\times 200$ (**B1**, **B2**, and **C**).

Statistical Analysis

All data are presented as means \pm SEM. Comparison of NMB-treated group and control group data was analyzed using one-way ANOVA. Comparison of NMB-treated group and siRNA+ NMB-treated group data was analyzed using two-way ANOVA. Pearson and linear analyses were performed for analysis of the relationship between genes and proteins. A P value of <0.05 was considered significant. Statistical analyses were performed using SPSS version 17.0 software for Windows (Microsoft).

RESULTS

Primary Cell Culture and Verification

Cultured primary SMCs within passage 4 were long and fusiform in shape. Cell cloning was performed after 48-h culture, and fusion was apparent in some clones 1 week later (Fig. 1A). Immunofluorescence and ICC results showed that the majority of cultured SMCs were long and fusiform or polygonal in shape. Intracytoplasmic green filamentous structures were observed, correlating with actin protein expression (Fig. 1, B1 and B2), thus confirming the identity of SMCs. ICC results confirmed that NMBR expression (Fig. 1C) in the cell membrane of the majority of cultured smooth muscle cell were positive. Thus, these results provide direct evidence that the cultured smooth muscle cell within passage 4 from the mice term myometrium demonstrate abundant expression of α -SMA and NMBR protein.

NMB-Induced DNA Binding Activity of Relap65 Blocked by Nmb-Specific siRNA Pretreated Myometrial Cells

Significant differences were observed among the binding activity of Relap65 DNA in pregnant SMCs treated with 10^{-8} M NMB, 10^{-6} M NMB, and 10^{-4} M NMB ($P < 0.05$). Higher binding activity was also detected in these three groups than in the control group ($P < 0.05$), but not in the 10^{-10} M NMB-treated group ($P > 0.05$). The binding activities of Relap65 DNA in Nmb siRNA+ NMB (10^{-10} M $\sim 10^{-4}$ M) treated

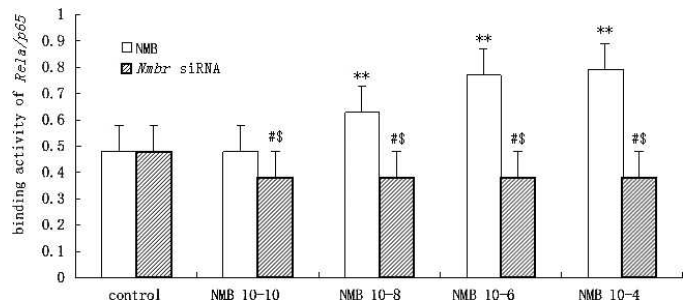


FIG. 2. Influence of NMB on the DNA binding activity of Relap65 in primary cultured pregnant myometrial cells. There were significant differences among the activity of Relap65 in 10^{-8} M $\sim 10^{-4}$ M NMB groups and control groups ($**P < 0.01$). There were no significant differences between the activity of Relap65 in 10^{-10} M NMB and control groups. After Nmb siRNA, there were significant differences among the activity of Relap65 in Nmb siRNA+ 10^{-10} M $\sim 10^{-4}$ M NMB groups and control groups ($*P < 0.01$) and that in the same concentration of NMB ($*P < 0.01$). There were no significant differences among four Nmb siRNA+ NMB groups.

groups were lower than that of the control group and in the same concentrations of NMB before siRNA pretreatment, respectively ($P < 0.05$). No significant differences were identified among the four groups pretreated by siRNA ($P > 0.05$) (Fig. 2). Thus, these findings demonstrate that 10^{-8} M $\sim 10^{-4}$ M NMB can up-regulate the DNA binding activity of Relap65, and this role is blocked by Nmb-specific siRNA in pregnant SMCs.

NMB-Induced Il6 Expression Reduced by Nmb- or Relap65-Specific siRNA Pretreated Myometrial Cells

Significant differences was observed among the levels of Il6 mRNA and protein in pregnant SMCs treated with 10^{-8} M NMB, 10^{-6} M NMB, and 10^{-4} M NMB ($P < 0.05$). Higher levels were also detected in these three groups than in the control group ($P < 0.05$), but not in the 10^{-10} M NMB-treated group ($P > 0.05$). The levels of Il6 mRNA and protein in Nmb siRNA+ NMB (10^{-10} M $\sim 10^{-4}$ M) groups were lower than that of the control group and that in the same concentration of NMB before siRNA pretreatment, respectively ($P < 0.05$). No significant differences were identified among the four groups pretreated by siRNA ($P > 0.05$) (Fig. 3A). The levels of Il6 mRNA and proteins in Relap65 siRNA+ NMB (10^{-10} M $\sim 10^{-4}$ M) treated groups were also lower than that in the control group and that in same concentration of NMB before siRNA pretreatment, respectively ($P < 0.05$). No significant differences were identified among the four siRNA groups ($P > 0.05$) (Fig. 3B), and no remarkable differences were detected between the effects of siRNA on Nmb and Relap65 on the levels of Il6 mRNA and protein ($P > 0.05$). Taken together, these findings provide strong evidence that NMB can induce the expression of Il6, and this up-regulating role is intercepted equally via Nmb- or Relap65-specific siRNA in pregnant SMCs.

Influence of Nmb- or Relap65-Specific siRNA Pretreatment on NMB-Induced $[Ca^{2+}]_i$ in Pregnant Myometrial Cells

A remarkable difference was observed in the levels of average or maximum $[Ca^{2+}]_i$ in pregnant SMCs among cultures treated with 10^{-8} M $\sim 10^{-4}$ M NMB jointly with extracellular calcium ($P < 0.01$). The differences were also shown between the joint group with the NMB or extracellular

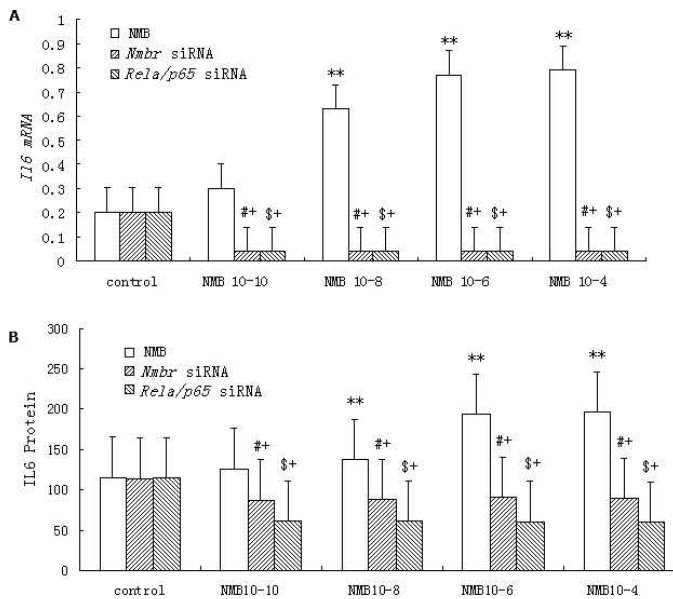


FIG. 3. Influence of NMB on the expression of *Il6* mRNA and protein in primary cultured pregnant myometrial cells. There were significant differences among the level of *Il6* mRNA and protein in 10^{-8} M $\sim 10^{-4}$ M NMB groups and 10^{-10} M NMB and control groups (** $P < 0.01$) and no significant differences between 10^{-10} M NMB and control groups. After we applied *Nmb* siRNA and *Rela/p65* siRNA, there were significant differences among the levels of *Il6*, both in 10^{-10} M $\sim 10^{-4}$ M NMB groups and control groups, respectively ($^{\#}P < 0.01$; $^{\$}P < 0.01$). There were lower levels of *Il6* than in the same concentration before siRNA treatment was performed ($^{+}P < 0.01$) and no significant differences between *Nmb* siRNA and *Rela/p65* siRNA in every NMB group.

calcium-alone treatment group or 10^{-10} M joint group ($P < 0.01$, respectively), but no differences were found within NMB- or CaCl_2 -alone treated groups ($P > 0.05$) (Fig. 4A). These findings demonstrate the role of NMB in inducing the increase of $[\text{Ca}^{2+}]_i$ in pregnant SMCs relies on the presence of extracellular calcium.

The levels of $[\text{Ca}^{2+}]_i$ in *Nmb* siRNA + NMB (10^{-10} M $\sim 10^{-4}$ M) treated groups were lower than that of the control group and that in same concentration of NMB before siRNA pretreatment groups, respectively ($P < 0.05$). No significant differences were identified among the four groups by siRNA pretreatment ($P > 0.05$). The $[\text{Ca}^{2+}]_i$ in *Rela/p65* siRNA + NMB (10^{-10} M $\sim 10^{-4}$ M) groups were lower than that in the control group and that in the same concentrations of NMB groups before siRNA pretreatment, respectively ($P < 0.05$). No significant differences were identified among the four groups pretreated by siRNA ($P > 0.05$) (Fig. 4, B and C). However, significant differences were observed in $[\text{Ca}^{2+}]_i$ between the *Rela/p65*-specific and *Nmb*-specific siRNA-treated groups ($P < 0.05$) (Fig. 4, B and C). Therefore, these findings showed that NMB (10^{-8} M $\sim 10^{-4}$ M) jointly with

extracellular calcium can dramatically increase the levels of $[\text{Ca}^{2+}]_i$, and this role is impaired by *Nmb*- or *Rela/p65*-specific siRNA in pregnant smooth muscle cells, but this regulating role is unbalanced between *Nmb* and *Rela/p65*.

Correlations Between the NMB-Induced DNA Binding Activity of *Rela/p65*, *Il6* mRNA Levels, and $[\text{Ca}^{2+}]_i$ in Smooth Muscle Cells Pretreated with *Nmb*-Specific siRNA

Positive correlations were identified between the DNA binding activity of *Rela/p65* and *Il6* mRNA levels ($r = 0.952$; $P < 0.01$) and between $[\text{Ca}^{2+}]_i$ and the DNA binding activity of *Rela/p65* and levels of *Il6* mRNA ($r = 0.278$; $P < 0.05$; and $r = 0.293$; $P < 0.05$, respectively) induced by a different concentration gradient of NMB (10^{-10} M $\sim 10^{-4}$ M) with joint extracellular calcium. However, this regulating relationship between levels of *Rela/p65* and *Il6* is not observed after *Nmb*-specific siRNA treatment was performed. Thus, these findings not only reveal the parallel relationship in the regulation of *Rela/p65* and *Il6* induced by NMB but also provide evidence that the induction of *Il6* by NMB up-regulated in the cultured SMCs within passage 4 from the pregnant myometrium requires the *Rela/p65* signaling pathway and extracellular calcium.

Correlations Between NMB-Induced $[\text{Ca}^{2+}]_i$ and *Il6* mRNA Levels and in SMCs Pretreated with *Rela/p65*-Specific siRNA

No correlations were identified between NMB-induced $[\text{Ca}^{2+}]_i$ and levels of *Il6* mRNA in SMCs pretreated with *Rela/p65*-specific siRNA ($P > 0.05$). Thus, these results suggested that the up-regulation of $[\text{Ca}^{2+}]_i$ induced by NMB via inflow from extracellular calcium is not in line with the regulation of *Il6* and can cross-talk with other pathways.

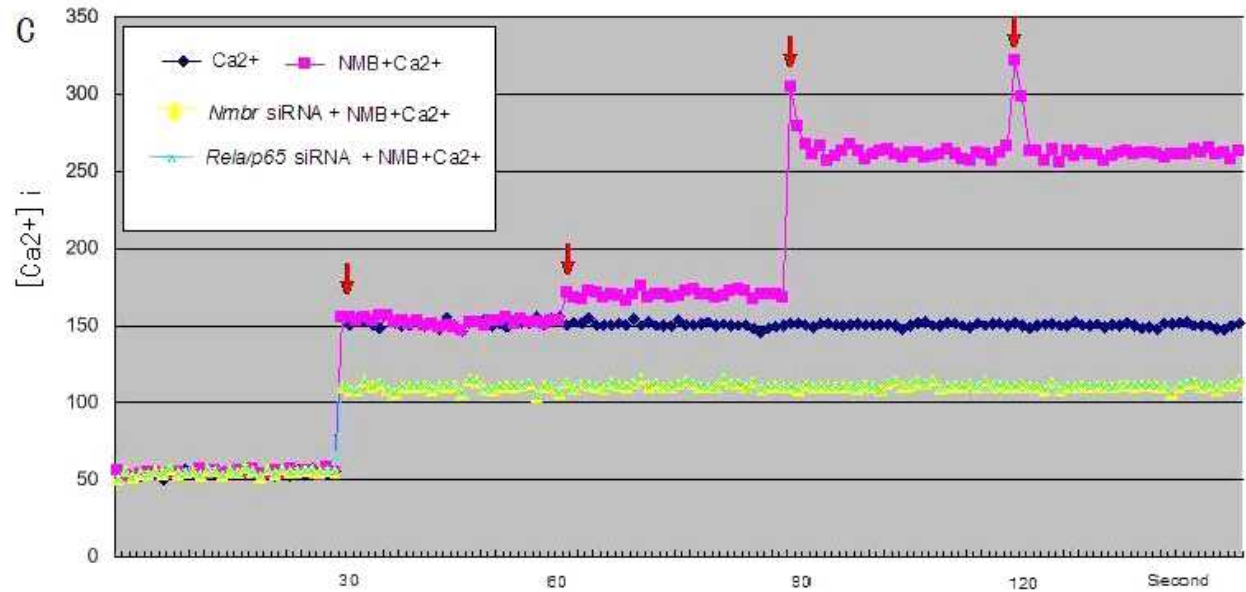
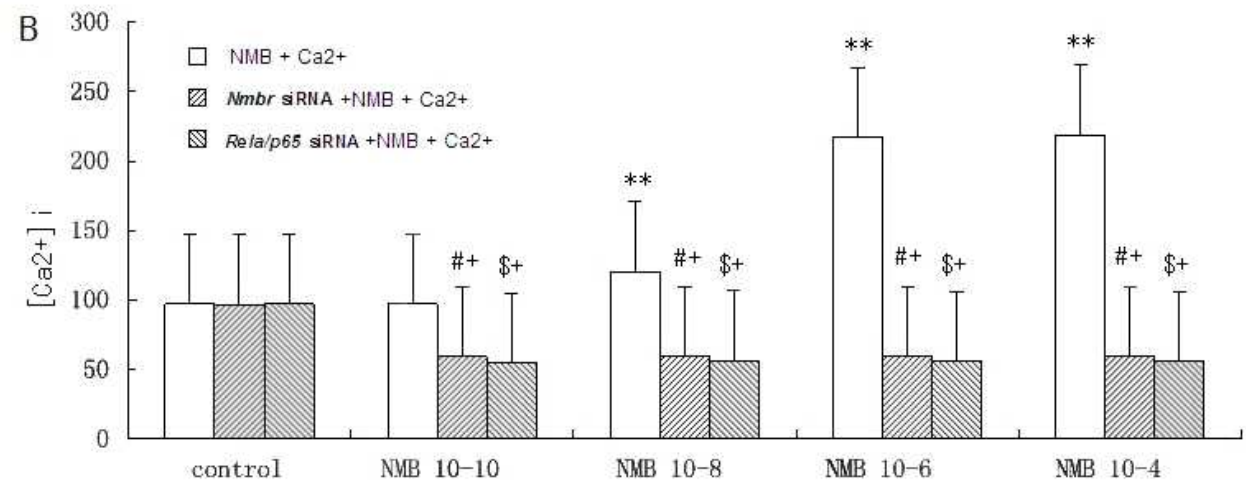
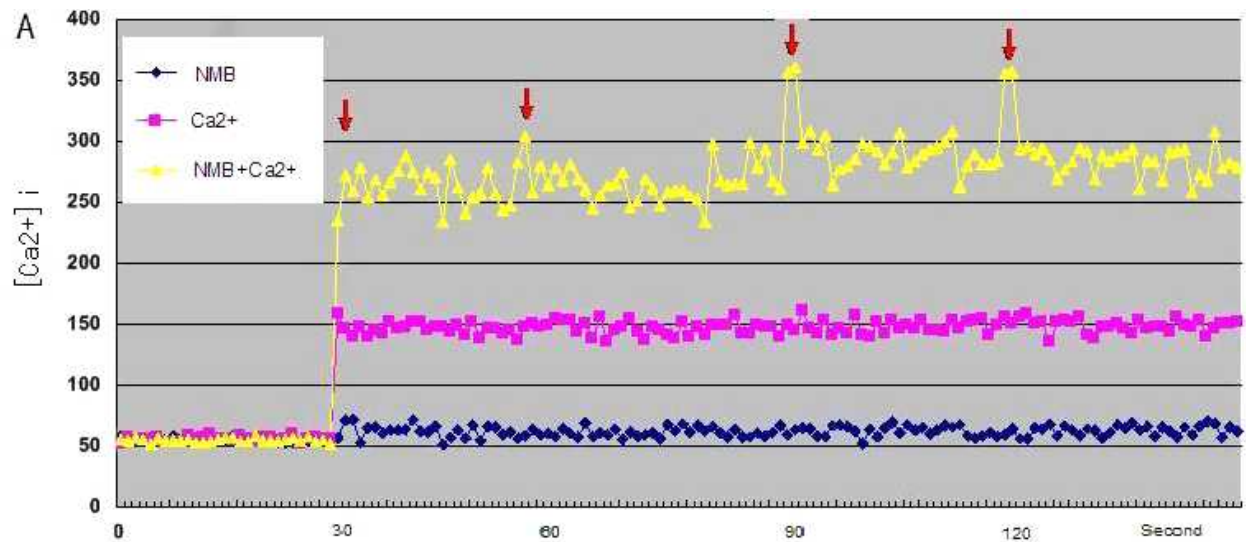
Comparison of the Influences of siRNA on *Nmb* and *Rela/p65* on NMB-Induced *Il6* and $[\text{Ca}^{2+}]_i$ Levels in SMCs

No significant differences were detected between the effects of siRNA on *Nmb* and *Rela/p65* on the levels of *Il6* mRNA ($P > 0.05$) (Fig. 3A). However, significant differences were observed in $[\text{Ca}^{2+}]_i$ between the *Rela/p65*-specific and *Nmb*-specific siRNA-treated groups ($P < 0.05$) (Fig. 4B). Overall, these results strongly indicate that regulation of *Il6* and $[\text{Ca}^{2+}]_i$ induced by NMB do not occur in the single signal pathway and can cross-talk with the other pathways.

DISCUSSION

Investigation of the molecular mechanisms involved in the *Nmb/Nmb* interaction in myometrial contraction in vitro is currently limited by the lack of availability of a suitable model cell line. However, primary cell cultures have been shown to retain morphological and functional characteristics [27].

FIG. 4. Influence of NMB on the levels of $[\text{Ca}^{2+}]_i$ in primary cultured pregnant myometrial cells. **A**) In the map of level $[\text{Ca}^{2+}]_i$, NMB could only cause slight fluctuation of the level of $[\text{Ca}^{2+}]_i$ and without concentration-dependent increase on the level of NMB; the extracellular calcium (CaCl_2) can increase the level of $[\text{Ca}^{2+}]_i$ to 150 optical units; the joint groups of NMB with extracellular calcium can increase dramatically the level of $[\text{Ca}^{2+}]_i$ from 250 to 350 optical units. **B** and **C**) Influence of NMB on the levels of $[\text{Ca}^{2+}]_i$ after and before siRNA. There were significant differences among the level of $[\text{Ca}^{2+}]_i$ in 10^{-8} M $\sim 10^{-4}$ M NMB groups and 10^{-10} M NMB and control groups (** $P < 0.01$) and no significant differences between 10^{-10} M NMB and control groups. After *Nmb* siRNA and *Rela/p65* siRNA treatments were performed, there were significant differences among the level of $[\text{Ca}^{2+}]_i$ both in 10^{-10} M $\sim 10^{-4}$ M NMB groups and control groups, respectively ($^{\#}P < 0.01$, $^{\$}P < 0.01$). There were lower levels of $[\text{Ca}^{2+}]_i$ than in same concentration before siRNA treatment was performed ($^{+}P < 0.01$).



Previous studies *in vivo* have shown that the expression level of *Nmbr* reached a peak at term and decreased sharply after labor [11]. Thus, this study was conducted using primary cultures of myometrial primary cells isolated at the onset of labor immediately after parturition of the first neonate or in the interval between delivery of two pups. Standard immunochemical staining techniques were used to demonstrate α_2 -actin and NMBR expression, thus confirming the myogenic origin of cultured cells. Furthermore, morphological characteristics and expression of NMBR were shown to be retained for at least four generations. Therefore, this primary myometrial cell culture constitutes a suitable model of the onset of labor for the investigation of the role and mechanism of the *Nmb/Nmbr* interaction in delivery, using RNA interference (RNAi) technology for the identification of genes involved in the regulation of this interaction.

The use of this model in this study revealed that *Rela/p65* and *Il6* levels induced by the *Nmbr* agonist NMB were increased in a concentration-dependent manner jointly with extracellular calcium. This is in accordance with previous reports of *Nmbr* agonist-mediated up-regulation of *Rela/p65* and *Il6* expression in a mouse model of pregnancy in addition to the down-regulatory role of *Nmbr* antagonists [28]. Increased DNA binding activity of *Rela/p65* is regarded as the key step in the onset of labor [18]. Moreover, the up-regulation of *Rela/p65* activity enhances gene transcription of proinflammatory cytokines including *Il6* and *Il8* [19–21], which are closely associated with the contraction of uterine smooth muscle and dilation of the cervix [22–24]. This study demonstrated that the regulatory role of the *Nmb/Nmbr* interaction on *Rela/p65* and *Il6* expression is blocked by siRNA-mediated *Nmbr* knockdown. Furthermore, this effect on *Il6* expression was also blocked by *Rela/p65* silencing. It was noted that both *Nmbr* and *Rela/p65* knockdown resulted in similar inhibition of *Il6* expression at the mRNA and protein level. Mouse and human *Nmbr* have been shown to be coupled to phospholipase C, resulting in a breakdown of phosphoinositides, mobilization of cellular calcium, and activation of protein kinase C [13–16]. Gastrin-releasing peptide (GRP) activation of its receptor (GRPR), another bombesin-like peptide and receptor, has also shown to result in PKC activation and to elicit increased $[Ca^{2+}]_i$ in cancer and neuroendocrine cells [16–17]. The activity of *Nmbr* in BALB/3T3 cells is governed by PKC activation [13], which is known to activate *Rela/p65*-mediated regulation of the expression of proinflammatory genes during the onset of labor [15]. These data suggest that the *Nmb/Nmbr* interaction regulates *Il6* expression via the *Rela/p65* pathway in primary cultured myometrial cells from pregnant mice.

In this study, $[Ca^{2+}]_i$ was induced by NMB with extracellular calcium jointly pretreated in a concentration-dependent manner that correlated with *Rela/p65* activity and *Il6* expression. However, this role can be reduced in the absence of extracellular calcium, and differences in the levels of $[Ca^{2+}]_i$ were not identified among the different concentration gradients of NMB groups. Moreover, the up-regulation of $[Ca^{2+}]_i$ by NMB was remarkably diminished by *Rela/p65*-specific and *Nmbr*-specific siRNA treatment. The significant differences were also noticed in levels of $[Ca^{2+}]_i$ in *Rela/p65*-specific and *Nmbr*-specific siRNA-treated groups. A higher inhibition level of $[Ca^{2+}]_i$ was detected in response to *Rela/p65*-specific knockdown than to *Nmbr*-specific knockdown. These results suggest that the NMB elicit increased $[Ca^{2+}]_i$ depending on the joint presence of extracellular calcium via PKC, and it may involve other pathways in *Nmbr*-mediated effects on myometrial cell activity. Related studies have shown

that *Nmbr* activation stimulates phospholipase A2 and phospholipase D via both PKC-dependent and independent mechanisms [29]. Phospholipase A2 activity is a crucial enzyme in the regulation of the production and release of prostaglandin, which induces myometrium contraction and cervix ripening [29, 30]. Moreover, *Nmbr* stimulation also results in activation of tyrosine kinases and tyrosine phosphorylation of p125FAK by a phospholipase C-independent mechanism which requires p21 and the integrity of the actin cytoskeleton [31]. *Nmbr* activation also stimulates tyrosine phosphorylation of paxillin and MAP kinase activation [32]. Moreover, native and transfected rat *Nmbr* exhibit similar binding and signaling characteristics in BALB/3T3 cells [13]. These results have clearly demonstrated that *Nmbr* is involved in parturition via a number of signaling pathways in addition to its function in the regulation of the nervous system [33–37].

In conclusion, this study of the molecular mechanisms of parturition has demonstrated that the *Nmb/Nmbr* interaction influences the activity of myometrial primary cells *in vitro* predominantly through regulation of *Il6* expression via the *Rela/p65* pathway. Furthermore, data also indicate that the effects of *Nmbr* on the $[Ca^{2+}]_i$ involve the joint presence of extracellular calcium, but those effects involving transmitted pathways remain to be elucidated.

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