Decreased DNA Methylations at the Progesterone Receptor Promoter A Induce Functional Progesterone Withdrawal in Human Parturition

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Abstract

The functional interaction of progesterone receptor (PR) isoforms PRA and PRB regulates myometrial transition from the resting state to excitation–contraction to initiate parturition. However, the regulatory mechanisms responsible for maintenance and functional alteration of the PRA and PRB expression levels during human pregnancy and term labor, respectively, remain unknown. Therefore, this study was designed to investigate whether and how epigenetic DNA modifications, specifically methylations, at the PRs' promoter regions contribute to the differential expression of PRA and PRB in laboring term myometrium of humans. Comparative analysis of PRA and PRB messenger RNA (mRNA) expression levels and accompanying changes in their promoters' methylation status was carried out using human myometrial samples from women undergoing singleton, term deliveries by cesarean section, either in the absence of labor (designated as NIL for not-in-labor) or in active labor (designated as IL for in labor). The *PRA* gene expression was shown to be elevated significantly during labor, while *PRB* gene expression was unaltered, and this differential expression was accompanied by decreased DNA methylation at the PRA promoter and not at the PRB promoter. In addition, labor-related decreased mRNA expression of the DNA methyltransferase (DNMT) family members DNMT1 and DNMT3a was found, however whether the increased expression of DNMTs directly supports the functional withdrawal of progesterone needs further investigation. Collectively, these data indicate that DNA methylation might represent an important epigenetic mechanism of labor-related differential expression of PRs, thereby mediating the biological process of functional PR withdrawal at term for parturition.

Keywords

progesterone receptors, myometrium, labor, DNA methylation, DNMT

Introduction

Successful pregnancy and delivery are dependent upon interactions of multiple hormones to regulate the uterine muscle tone. Throughout pregnancy, the resting state of the organ's smooth muscle is maintained by progesterone through its myriad interactions with other hormones.¹ Transition from the resting state to excitation–contractions initiates the labor process. Although this phase is accompanied by a dramatic and rapid reduction in maternal plasma progesterone levels in most nonprimate mammalian species,² in humans the circulating progesterone levels remain high but circulating estrogen levels increase remarkably.³ This human hormone profile, however, seems to contradict the robust and consistent clinical success of RU486 (generic name: mifepristone), an antagonist of progesterone receptors (PRs), as an inducer of uterine contractions during pregnancy that culminate in

labor^{4,5}; it is important to note that this synthetic steroid compound is used mainly in cases of nonlive births. The concept of functional progesterone withdrawal emerged as a possible

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explanation for the inducer activity of RU486 and the apparent functional irrelevance of progesterone during natural labor.

Both PRA and PRB, the 2 predominant isoforms of the human nuclear PRs, act as transcription factors upon binding to their cognate ligands (primarily progesterone). The mechanisms of activated PRB are associated with the resting state of the uterine smooth muscle, while those of PRA have been characterized as inhibitive of the PRB effects. Clinical observations of humans have revealed that the myometrial PRA expression becomes increased relative to PRB expression at term labor, and related studies have indicated that this differential PR isoform ratio suppresses the responsiveness to myometrial progesterone, thereby triggering labor and delivery.⁶⁻⁹ Yet, the mechanisms regulating the expression of the PR isoforms remain largely unknown.

DNA methylation and/or histone acetylation in the promoter regions of genes regulate transcription by modulating chromatin conformation.^{10,11} Hypermethylated DNA and deacetylated histones are usually associated with downregulated or silenced gene expression.¹² In contrast, hypomethylated DNA and acetylated histones are associated with upregulated or activated gene expression. Altered DNA methylation in the genes encoding PRs has been observed in numerous human cancers; for instance, hypermethylation was detected in prostate carcinomas¹³ and in specimens of breast and endometrial cancers with downregulated PR expression.¹⁴⁻¹⁶ Moreover, induced hypermethylation of the PRB promoter in endometrial cancer cell lines was shown to silence the *PRB* gene expression, and this effect was reversed upon induction of demethylation in conjunction with histone acetylation.¹⁷ Intriguingly, a recent study of PRA and PRB expression in human term myometrium found that the respective promoters of both isoforms had higher levels of histone acetylation and H3K4me3 than those observed in preterm myometrium,¹⁸ providing the first evidence of a potential epigenetic mechanism mediating the functional withdrawal of progesterone.

The present study was designed to further investigate whether epigenetic DNA methylations are involved in regulation of the myometrial expression of PR isoforms, in order to gain insights into the epigenetic-related mechanisms of normal labor. The results indicated that during normal labor, *PRA* gene expression is elevated significantly but *PRB* gene expression is not altered. The increased *PRA* gene expression was associated with decreased DNA methylation in its promoter and accompanied by increased gene expression of both DNA methyltransferase (DNMT) 1 and DNMT3a. Therefore, DNA methylation might represent an additional epigenetic mechanism mediating the functional withdrawal of progesterone at term through regulation of PRs' gene expression; however, whether the increased expression of DNMTs directly supports the functional withdrawal of progesterone needs further investigation. Identifying the epigenetic mechanisms, such as DNA and histone modification,¹⁸ that underlie term labor may provide molecular targets of progestin-based interventions to prevent preterm birth.

Materials and Methods

Tissue Specimens

All uterine tissue specimens used in this study were obtained from women attending the Southwest Hospital for standard (not study related) care. The study was carried out with pre-approval from the hospital's Ethical Research Committee, and all enrolled women provided written informed consent for tissue donation and analysis and for publication of findings.

The myometrium tissue biopsies ($0.5 \times 0.5 \times 0.5 \text{ cm}^3$) were collected from the upper margin of the lower uterine segment of women undergoing singleton, term deliveries by cesarean section, either in the absence of labor (designated as NIL for not-in-labor) or in active labor (designated as IL for in labor). The NIL cesarean deliveries were elective and offered based upon patient history of previous cesarean section and/or breech presentation; for these women, no signs of labor onset, such as uterine contractions or cervical changes, were evident at the time of surgery. The IL cesarean deliveries were performed under emergency circumstances for unresolvable breech presentation or fetal distress.

All tissue samples were submerged in ice-cold phosphate-buffered saline (PBS), diced into small pieces, and divided into 4 for immediate processing as follows: 1 portion was mixed with fixative (4% paraformaldehyde in PBS), incubated at 4°C for 24 hours, and embedded in paraffin for subsequent histological analysis; 1 portion was used for RNA extraction; 1 portion was used for DNA extraction; and the final portion was stored unmanipulated at -80°C.

RNA Extraction, Reverse Transcription, and Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted using the TriPure Reagent (Roche, Indianapolis, Indiana) and following the manufacturer's protocol. RNA concentration and quality were determined by spectrophotometric analysis and agarose gel electrophoresis, respectively. A 500-ng aliquot (in RNase-free double-deionized [dd] H₂O; Takara, Japan) was reverse transcribed into complementary DNA using the Takara PrimeScript RT Reagent kit (Perfect Real Time, Shiga) and applied as template for polymerase chain reaction (PCR) amplification (40 cycles of 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 20 seconds) with gene-specific primers (Table 1) and SYBR Premix ExTaq II, according to the manufacturer's instructions. The threshold cycle values were normalized against a threshold value of β -actin (reference gene). The relative abundance of PRA messenger RNA (mRNA) was calculated by subtracting the relative abundance of PRB mRNA from that of PR total.

Immunohistochemistry

Two anti-PR antibodies, one specific to PRB and the other to PRA/B (both from Leica, England), were used to identify PRB and PR total protein expression in the NIL and IL uterine

Table 1. Primers Used in This Study.

Gene Target	Forward Primer, 5'→3'	Reverse Primer, 5'→3'
qRT-PCR primers		
PR total	AGCCCACAATACAGCTTCGAG	TTTCGACCTCCAAGGACCAT
PRB	AACTCAGCGAGGGACTGAGA	GAGGACTGGAGACGCAGAGT
DNMT1	GCACCTCATTGCGGAATACA	TCTCCTGCATCAGCCCAAATA
DNMT3a	GCACTGAAATGGAAAGGG	TAAGAGGTGGCGGATGACT
DNMT3b	CCATGAAGGTTGGCGACAA	TGGCATCAATCATCACTGGATT
β-actin	CCATGTACGTTGCTATCCAGG	TCTCCTTAATGTCACGCACGA
BSP primers		
PRA-B	TGATTGAGTTGAAGGTAAAGGGT	TCCAACACCCCTTATAACTTCAA
PRB1-B	TGTGGGTGGTATTTTTAATGAGAA	CCCTCACTAAAACCCCTAAAATAAT
PRB2-B	TGAGGGATTGAGAGTTTTATAGTATGTA	CAACTCCTACCCTTAACCTCCAT

Abbreviations: PR total, primers for amplification of both the *PRA* and *PRB* genes; PRAB, BSP primer for the *PRA* promoter; PRB1/2-B, BSP primer for the 2 CpG islands in the *PRB* promoter; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; PR, progesterone receptor; DNMT, DNA methyltransferase; BSP, bisulfite DNA sequencing.

smooth muscle tissues by standard immunohistochemical techniques. Because it is not possible to raise an antibody specific to *PRA*, the immunohistochemical analysis of the *PRA* expression was conducted by subtractive inference. The paraffin-embedded uterine myometrium blocks were cut into 4 μm sections and dried at room temperature. After deparaffinization and rehydration, sections were treated with 3% hydrogen peroxidase in methanol for 20 minutes to inactivate endogenous peroxidase. Antigen retrieval was carried out by autoclaving (121°C, 18 psi pressure) the sections for 4 minutes in an EDTA antigen unmasking solution (ZSGB-Bio, China). After blocking with normal goat serum solution for 30 minutes (ZSGB-Bio), the sections were incubated with 1 of the 2 primary antibodies (1:100 *PRA/B* or 1:50 *PRB*, diluted in PBS) at 4°C overnight, in a humidified chamber. Sections were washed with PBS (pH 7.8) and then incubated with the appropriate secondary antibody solution (ZSGB-Bio). Immunostaining was detected by the avidin–biotin–peroxidase method (ZSGB-Bio) with diaminobenzidine as the chromogen and followed by counterstaining with hematoxylin.

Bisulfite DNA Sequencing

Genomic DNA was isolated from the NIL or IL myometrium tissues using a genomic DNA isolation kit (Promega, Sunnyvale, CA) and following the manufacturer's protocol. A 500-ng aliquot of the purified DNA was denatured using NaOH and treated with sodium bisulfite (Sigma, St. Louis, MO) for 16 hours, as described previously.¹⁹ The modified DNA was desalinated and purified, ethanol precipitated, and resuspended in 30 μL ddH₂O. DNA methylation of CpG island in the promoter region of *PRA* or *PRB* gene was analyzed using the bisulfite DNA sequencing (BSP) method. The bisulfite primers were designed using the Methyl Primer Express v1.0 software (Life Technologies Co, New York) and listed in Table 1. Each of the DNA samples was amplified by PCR as follows: 95°C for 5 minutes; 10 cycles of 95°C for 30 seconds, 60°C to 50°C (Δ1°C) for 45 seconds, and 72°C for 45 seconds; 33 cycles of 95°C for 30 seconds, 50°C for

45 seconds, and 72°C for 45 seconds; and 72°C for 10 minutes. Amplified products were purified with the QIAquick Gel Extraction kit (Qiagen, Germantown, MD) and cloned into the pCR2.1 vector (Life Technologies Co), and individual clones were sequenced using M13 reverse primer and an automated ABI Prism 3730xl Genetic Analyser (Applied Biosystems, Foster city, California). Ten clones from each PCR product were sequenced to characterize the methylation status of the CpG sites. The schematic diagram of the CpG island location and the CpG amplification primer fragment in the promoter region of *PRA* and *PRB* genes are shown in Figure 1.

Statistical Analysis

All mRNA abundance data are expressed relative to the β-actin reference gene. The relative mRNA abundance was calculated using the $2^{-\Delta Ct}$ formula, where *Ct* is the mean threshold cycles of PCR in triplicate, and Δ*Ct* is the *Ct* difference between the reference and target mRNA.¹⁸ All data were initially tested for normality using a Student *t* test for 2 groups.

Results

PRA Gene Expression is Increased in Uterine Smooth Muscles During Human Labor

The quantitative real-time reverse transcription PCR (qRT-PCR) assay showed that, unlike the *PRB* mRNA levels, the *PRA* mRNA levels were markedly higher in IL myometrium than that in NIL myometrium (Figure 2A). More importantly, the *PRA-PRB* mRNA ratio was significantly higher in the IL myometrium (Figure 2B).

Immunohistochemistry analysis of the NIL and IL myometrial samples indicated that the protein expression of total PR was elevated markedly during labor (Figure 3). Since no significant change in *PRB* was observed, the total PR increase represents a specific increase in *PRA* protein expression during labor.

Figure 1. The CpG island and CpG amplification primer fragment in the promoter of *PRA* and *PRB* genes. Schematic diagram showing the transcription start site of *PRA* and *PRB* genes. The CpG island location and the CpG amplification primer fragment in the promoter region of *PRA* and *PRB* genes were indicated, respectively. PR indicates progesterone receptor.

Figure 2. Myometrium mRNA expression of *PRA* and *PRB* prior to and after labor onset. Panel A, relative mRNA abundance of *PRA* and *PRB* in human myometrial samples collected prior to labor onset (NIL, $n = 25$) or after labor onset (IL, $n = 25$), expressed relative to the β -actin reference gene. Panel B, *PRA*-*PRB* mRNA expression ratio prior to and after labor onset ($n = 25$). Data are presented as mean $\pm$ SD. * $P < .05$ versus the corresponding NIL group; ** $P < .01$ versus the NIL group. mRNA indicates messenger RNA; PR, progesterone receptor; NIL, not in labor; IL, in labor; SD, standard deviation.

Methylation of *PRA* Gene Promoter is Significantly Decreased in Myometrium During Labor

DNA methylation of the CpG islands within a gene promoter region is associated with decreased gene expression¹²; therefore, we detected the DNA methylation status in the gene promoters of *PRA* and *PRB* by BSP assay to dissect the epigenetic mechanisms responsible for the regulation of their expression. The *PRA* gene promoter showed a high level of methylations in the NIL specimens, and the IL specimens showed a significantly lower level of methylations in this gene region (Figure 4).

The *PRA* promoter region contains only 1 CpG island, while the *PRB* promoter region contains 2 CpG islands (Figure 1); therefore, we analyzed the overall *PRB* promoter methylation status by combining the results from the 2 CpG Islands. As

shown in Figure 4, the NIL specimens contained much higher levels of methylation in the *PRB* gene promoter than the IL specimens, suggesting that the labor event did not affect (promote) methylation in this gene region. In general, the promoter methylation status detected for the 2 *PR* genes was in agreement with the detected expression levels.

DNA Methyltransferase Genes' Expression Levels Mirror Labor-Related Changes in Expression of *PRA* and *PRB* Genes

To date, 3 members of the *DNMT* gene have been identified in mammals: *DNMT1*, *DNMT2*, and *DNMT3*. Although the function of *DNMT2* remains unclear, *DNMT1* and *DNMT3* (including the *DNMT3a* and *DNMT3b* isoforms) have been defined as

Figure 3. Protein expression of PRs during labor. PR total (left) and PRB protein (right) expression (brown) is shown in NIL and IL myometrium tissues. The color version of this figure is available online at rs.sagepub.com. PR indicates progesterone receptor; NIL, not in labor; IL, in labor.

Figure 4. DNA methylation of PRA and PRB during labor. Methylation status of PRA (left) and PRB (right) is shown. The 2 methylated CpG sites in PRB were summed and presented as total CpG islands for the gene region. Data are plotted as mean ($n = 6$) $\pm$ SD. ** $p < .01$ versus the NIL group. SD indicates standard deviation; PR, progesterone receptor; NIL, not in labor.

important regulators of the mammalian methylation process^{20,21}; therefore, the potential differential expressions of *DNMT1* and *DNMT3* in NIL and IL myometrial specimens were investigated by qRT-PCR and compared to the labor-related differential expression patterns of the PRA and PRB. The expression of *DNMT1* and *DNMT3a* was found to be significantly lower in the IL specimens than in the NIL specimens (Figure 5); however, the NIL and IL specimens showed no significant difference in *DNMT3b* expression (Figure 5).

Discussion

Progesterone plays a central role in the maintenance of pregnancy and the initiation of parturition by modulating myometrial contractility and excitability. Several mechanisms of progesterone withdrawal at term have been described by various studies and include changes in transcription factor coactivators,^{22,23} nuclear factor- κ B,²³ and metabolism of progesterone by the target tissues.²⁴ Studies of the functional consequence of progesterone

Figure 5. The mRNA expression of DNMTs in myometrium during labor. The relative mRNA abundance of DNMT1 (right) and DNMT3a and DNMT3b (left) in human myometrial samples is shown. Relative expression was determined by comparison to the β -actin reference gene. Data are plotted as mean ($n = 25$) $\pm$ SD. * $P < .05$ versus the corresponding NIL group. mRNA indicates messenger RNA; DNMT, DNA methyltransferase; SD, standard deviation.

withdrawal have indicated a role for PRs; specifically, decreased PRA and PRB have been shown to accompany loss of myometrial progesterone responsiveness,^{3,9} while increase in the PRA-PRB mRNA ratio in myometrium has been shown to accompany the laboring process. In addition, progesterone responsiveness has been shown to depend on the level and the ratio of these PR isoforms, which exert their transcriptional regulatory functions on distinct sets of genes, and even on each other (ie, high levels of PRA act as a dominant inhibitor of PRB transcriptional activity).^{19,25-29}

The current study of women during parturition revealed a labor-associated increase in myometrial levels of PRA mRNA and in the PRA-PRB ratio but no change in the PRB levels. These findings agree with a previous study of functional progesterone withdrawal in rhesus monkeys, which found similar patterns of increased PRA-PRB ratio in the myometrium when measured directly after labor.³⁰ Since previous clinical reports have demonstrated differential methylation of the PRB promoter region accompanying changes in physiological conditions, such as endometrial cancer¹⁶ and breast cancer,³¹ and related (at least partially) to decreased *PRB* gene and protein expression, we hypothesized that the labor-related changes in myometrial expression of PRs may involve an epigenetic regulatory mechanism, such as DNA methylation. We found that the methylation of myometrial PRA was markedly lower during labor and delivery (vs nonlaboring myometrium during delivery), but that of PRB was not significantly different—agreeing with the labor-related differential expression patterns of mRNA. The decrease in PRA promoter methylation level and increase in DNMT1 and DNMT3a may explain the increased expression level of this gene and its role in functional progesterone withdrawal.

Studies of the mammalian DNMTs have suggested that DNMT1 acts as the foremost contributor to the methylation regulatory network,^{20,21} with its primary role involving

maintenance of the DNA methylation landscape upon cell division. In contrast, the DNMT3a family member plays a key role in de novo DNA methylation.³² Thus, the labor-related decreases in DNMT1 and DNMT3a expression observed in the current study suggest that the labor-related change in PRA expression (and presumably availability for functional interaction with its ligands) may be regulated, at least partially, by the methylation epigenetic mechanism; although, it is likely that other epigenetic mechanisms, such as acetylation, may play important roles as well. Certainly, these novel results indicate that the molecular process of mammalian parturition is highly complex, involving myriad signaling pathways and dynamic interplay of various hormones and enzymes; further studies based upon the differential expression and regulation of PRA revealed in this study will expand our understanding of this critical reproductive process and provide insights into labor-related complications and their prevention or resolution.

Conclusions

This study has provided the first reported evidence of term labor-related differential DNA methylation at the *PRA* gene promoter in human myometrial tissue. Specifically, *PRA* gene expression was shown to be elevated significantly during labor, while *PRB* gene expression remained unaltered, resulting in a marked enhancement in the PRA-PRB expression ratio and presumably affecting the functional withdrawal of progesterone that is necessary for initiating excitation-contraction and natural vaginal delivery. The regulatory mechanisms underlying this molecular process appear to involve inducement of differential DNA methylation in the PRA promoter region.

Authors' Note

Xia Li and Cheng Chen contributed equally to this work.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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