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Review Article

Molecular analysis of parturition via oxytocin receptor expression

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Abstract

In daily obstetric practice, oxytocin is one of the most frequently used drugs to stimulate uterine contraction. However, uterine sensitivity to oxytocin is profoundly different among pregnant women, which may reflect oxytocin receptor (OTR) expression in the uterus. We review here the literature focusing on OTR regulation in the human uterus. Recent progress in molecular biology has augmented our knowledge about OTR regulation in the uterus at the transcription level, although the totality of preparation for labor through OTR expression is still obscure.

Keywords: expression; oxytocin receptor; parturition

Introduction

After conception and implantation of the embryo, the uterus remains quiescent until the onset of labor and contractile forces cause expulsion of the fetus. Disorders of timing of labor onset, either preterm or post-term, may cause severe damage to the baby. Disorders of the force of labor, either too weak or too strong, may also cause dystocia, fetal asphyxia, or uterine rupture and hurt both mother and baby severely. Therefore, the regulation of timing and force of labor is critical for the safety of both mother and fetus.

Oxytocin is one of the most potent uterotonic reagents and is widely used in daily practice to induce or augment labor, and to treat *postpartum* hemorrhage. Oxytocin had been considered to be the major determinant of labor onset from its ability to induce labor at term; however, its concentration in maternal blood during the natural course of parturition does not increase until the second phase of labor [1]. The outrider works from Soloff et al [2] and Fuchs et al [3] indicate that oxytocin receptor (OTR) expression drastically increases just prior to onset of labor using ligand-binding assays with tritium-labeled oxytocin both in rat and human myometrium. These findings suggest that the upregulation of OTR expression may determine the timing of parturition via increased bioavailability of oxytocin. In order to prove this hypothesis, molecular analysis of the OTR may be necessary. It is well known that the oxytocin-OTR system plays various roles, not only in the uterus and mammary gland, but also in the central nervous system, behavioral state, kidney, heart, etc. [4,5]. Our review here focuses on the OTR regulatory mechanism in the uterus to stimulate investigation into the physiology of obstetrics.

Molecular analysis of OTR

In order to investigate the regulatory mechanism of a protein, it was first recognized that knowledge of its gene structure is necessary. However, in the early 1990s, there was no available information or database about OTR-related molecules. It was observed that the content of messenger (m)RNA encoding OTR in the human uterus at parturition was extremely high using a *Xenopus laevis* oocyte expression system [6]. This system was applied to cDNA library screening and human OTR cDNA was cloned [7]. Deduced amino acid sequence indicated that OTR belonged to the G-

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protein coupled, 7 transmembrane receptor family. OTR showed highest homology to the vasopressin V1a, V1b, and V2 receptors, which are now termed the nonapeptide hormone receptor family because oxytocin and vasopressin are highly homologous peptide hormones consisting of 9-amino acids. Using this cDNA as a probe, human OTR gene was investigated [8]. This gene spans approximately 17 kb, contains 3 introns and 4 exons, and located in chromosome 3p26.2. After the initial work, the gene structures of OTR were identified in other species such as rats [9], cattle [10] and mice [11], and their structures were compared (reviewed in [12]). Using this cDNA, it was confirmed that mRNA from human term uterine myometrium contains a large amount of OTR message. As this result paralleled the amount of OTR protein by western blotting, it was speculated that OTR expression was mainly regulated at the transcription level [13]. It was also determined that the expression of OTR increased after onset of labor as well as during the course of parturition in decidua [14]. It has been reported that oxytocin peptide itself is also produced in decidua and is increased after onset of labor [15]. Moreover, oxytocin stimulates prostaglandin secretion from human decidua [16] and prostaglandin levels in maternal plasma after successful induction by oxytocin were significantly higher than in those who failed to respond to oxytocin [17]. Taken together, there may be an oxytocin autocrine–paracrine circuit system in decidua and myometrium to induce and facilitate labor *in situ*. It makes sense that plasma oxytocin levels do not alter prior to the onset of labor if this system is mainly regulated within decidua *in situ*. Increased mRNA levels encoding OTR and other uterine activation proteins such as cyclooxygenase-2 (COX-2) or PGF2α receptor (FP) in decidua after onset of labor was observed in the uterine tissues during term and preterm labor [18], suggesting that the basic regulation for uterine activation was similar between term and preterm labor.

A recent report indicated that there was no difference in OTR mRNA expression between not-in-labor and in-labor myometrium [19]. However in this report, myometrium was obtained from cesarean delivery incision sites, equivalent to the lower segment of the uterus. As the gene expression pattern is very different between the fundus and lower segment of the uterus at parturition [20] and as the lower segment is often extended at term of pregnancy, this result might be caused by a sampling effect.

Regulation of OTR by estrogen

OTR is induced by estrogen. In rats and sheep, females whose ovaries had been removed lost the receptor expression and supplementation of estrogen recovered its expression in the brain and uterus. In late pregnancy rats, administration of tamoxifen, a selective estrogen receptor modulator, attenuated OTR expression in myometrium [21]. The upstream of the OTR genes was cloned in several species. In rats there is an incomplete palindromic estrogen responsive element sequence (ERE) 4 kb upstream of the gene [22]. The function of this element under estradiol treatment is obscure. All of the OTR gene upstream sequences contain several half sites of ERE, and tandem half sites of ERE are functional in the chicken ovalbumin gene [23]. However, when estrogen receptor (ER)α and reporter gene were cotransfected with human OTR upstream up to 7 kb into HeLa cells, no transactivating activity for estradiol was observed (T. Kimura, unpublished data). The OTR gene of many species contains not only ERE-half sites, but also SP-1 sites, nuclear factor (NF)-interleukin (IL)6 binding sites, stat-3 binding sites, AP-1 sites, etc. Telgmann et al made reporter constructs far upstream of the bovine OTR prompter region (up to −3.2 kb). Although they noticed other ERE-half sites in the far upstream region, estradiol did not show any effect on OTR expression in an ovine glandular epithelial cell line, which expresses endogenous ERs. After cotransfection with cyclic AMP response element binding protein-binding protein cDNA and SRC1 and/or SRC2 cDNAs, estradiol revealed significant transactivation activity [24]. The authors did not determine specific sequence elements that affect the estrogen transactivation. Precise interaction between OTR gene, ERα and these cofactors remains to be elucidated. In the ovine (sheep) model, short OTR promoter (up to −220 bp) revealed basal promoter activity and estrogen dependency under cotransfection with ovine ERα. When GC-rich SP-1 binding elements of the OTR gene were mutated, enhancement of promoter activity by estradiol was abolished. There was no ERE-half site within this region. Instead, it was noticed the progesterone receptor element half sites. Indeed when progesterone receptor (PR)B cDNA (see below) was cotransfected and stimulated with progestin, OTR promoter activity was facilitated [25]. This observation appears to be different from the physiological scenario, in which progestin usually suppresses OTR expression. In myometrium of relaxin (*Rlx*) knockout mice, both OTR and ERα expression were attenuated in late pregnancy [26], although pregnant female *Rlx*−/− mice gave birth normally. It is obvious that estrogen facilitates uterine OTR expression *in vivo*, although it appears not to be a direct effect. In term myometrium, rapid turnover of ERα protein was reported. When myometrial explants were stimulated by estradiol, extracellularly regulated kinase (ERK1/2) phosphorylation was observed, and phosphorylated ERK1/2 induced OTR mRNA expression [27]. This report did not reveal direct interaction between ERKs and the OTR gene. Any complex transcriptional machinery or nongenomic effects between estrogen and OTR should be further elucidated.

Regulation of OTR by progesterone

Progesterone acts to maintain uterine quiescence during pregnancy. Indeed, pregnant women with high risk of preterm birth can be effectively treated by progestin administration (reviewed in [28]). In rodents, luteolysis and *progesterone withdrawal* during pregnancy caused parturition. In sheep, increased cortisol from fetal adrenal gland steeply increased the estrogen/progesterone ratio in maternal blood via placental metabolism, and caused parturition (reviewed in [29]). However in humans, progesterone withdrawal occurs after delivery of the placenta [30], which is the major source of

progesterone synthesis. There are two isoforms for PR: PRA and PRB. PRA is the short truncated form of PRB and works as the dominant transactivation repressor of PRB in the presence of progestin. In human myometrium, PRA expression was reported to be higher after the onset of labor [31]. Induction of PRA protein functions may act as a progesterone withdrawal effect. In a nonlabor biopsy sample of human myometrium taken during cesarean delivery, the PRA/PRB mRNA ratio clearly correlated to the amount of ERα, COX-2, as well as to OTR mRNAs determined by quantitative RT-PCR [32]. It also strongly suggests that "functional" progesterone withdrawal in human myometrium regulates the OTR and other uterine activation proteins.

Another consideration is the anti-inflammatory effect of progesterone. RelA (p65), a component of NFκB, which is the representative NF mediating inflammatory events to gene transcription, directly interacts with PRB and PRA. Tumor necrosis factor (TNF)-α induced NFκB-mediated transactivation was attenuated by PRB, and progesterone induced PRB transactivation was repressed by TNF-α induced NFκB [33]. These *transrepression* effects of NFκB and PRB may also explain functional progesterone withdrawal. Plenty of clinical and experimental findings have shown the interaction between inflammation and uterine contraction. In the mouse model, administration of proteasome inhibitor to attenuate NFκB activation could delay the onset of antiprogestin-induced preterm birth [34]. This observation also suggests the interaction of PR function and NFκB *in vivo*. The transrepression theory of PR and NFκB is very attractive to explain functional progesterone withdrawal and uterine activation at parturition.

Recently, microRNAs (miRs) are considered to be another modulator for many genes. In mouse and human myometrium, miR-200 family members are increased at term and progesterone withdrawal by antiprogestin-induced miR-200 family expression. The miR-200 family interacts with mRNAs for ZEB1 and ZEB2, which are zinc-finger E-box-binding homeobox proteins, and act as transcriptional repressors, downregulating ZEB1 and ZEB2 expression. ZEB1 and ZEB2 inhibit the expression of the contraction associated protein genes including OTR [35]. Although there is no direct proof that the ZEB proteins interact with the OTR gene, upregulation of miR-200 family microRNAs by progesterone withdrawal facilitates uterine contraction. This observation might explain another pathway for how progesterone regulates uterine activity during pregnancy.

Inflammation, mechanical stimuli and the OTR

Causes of preterm birth are complex; however, clinical and experimental data indicate that infections (chorioamnionitis, intrauterine infection, and general infections such as pyelonephritis or pneumonia) and overstretching of the uterine wall (multiple pregnancy, polyhydramnios) could induce preterm birth. After cloning of the OTR gene promoter, researchers focused on the existence of tandem acute phase response element (now termed stat-3), NF-IL-6 and activator protein-1 consensus sequences and hypothesized their function at inflammatory stimuli. When IL-1β and IL-6 were applied to a myometrial cell line (ULTR), OTR mRNA level was decreased. CAT reporter activity, with the gene construct of the human OTR gene promoter region (−1203/+108), was attenuated by IL-1β and IL-6, although NF-IL-6 was induced in the nucleus of ULTR cells [36]. This observation was unexpected from clinical viewpoints and several other experiments were performed. It was reported that IL-1β decreased and IL-6 increased OTR mRNA expression [37–39], but a direct interaction between cytokine-induced nuclear factors and the OTR gene was not revealed. Using myometrial cell culture obtained from cesarean delivery, IL-1β revealed a biphasic effect on OTR expression, i.e., OTR was upregulated after 4 hours and downregulated after 20 hours. NFκB and CCAAT/enhancer-binding protein-b (C/EBPb) were activated after 4 hours of IL-1β stimulation, and they bound to NFκB and C/EBPb binding sites in the OTR gene promoter. Cotransfection of NFκB p65 (RelA) and C/EBPb massively activated the reporter activity with the OTR promoter [40]. RelA and C/EBPb interact each other in the nucleus and they bind together to the binding 20-bp element of −712 to −692 of the human OTR gene promoter [41].

Mechanical stretching of the human myometrial strip obtained during cesarean delivery before onset of labor, enhanced OTR mRNA expression. This stretching effect was abolished in similar samples obtained after onset of labor, probably because they were already stimulated by natural stretching due to labor. *In vitro* stretching of cultured human uterine myocytes activated AP-1, C/EBPb, but not NFκB. Transfection of C/EBPb cDNA into myocytes augmented OTR promoter activity in cultured myocytes, but AP-1 did not affect the OTR promoter [42].

Taken together, inflammatory and stretch signals may stimulate OTR promoter activity via C/EBPb; however, these effects have only been revealed by cotransfection type experiments.

Term myometrium-specific OTR gene binding protein: Differential protein-DNA binding analysis

In order to determine the myometrium-specific OTR upregulation mechanism at term, another straightforward strategy is comparison of DNA binding protein profile extracted from nonpregnant and term pregnant myometrium to the OTR promoter region. Nuclear proteins from nonpregnant and term myometrium were at first compared by gel-shift assay with a series of OTR promoter fragments, and shifted signal were further analyzed by methylation interference footprinting assay. Two binding elements that were not related to known nuclear protein-binding sites were investigated. The first element, US-1, was located within −1745 to −1729 bp of the human OTR promoter and showed enhancer activity. However, the study failed to identify a binding protein by a batch purification procedure [43]. The second element, US-2 (−1433 to −1414 bp of human OTR promoter), was applied to yeast one hybrid system as a bait and a cDNA library from human term myometrium was screened. A 2.3-kb full-length cDNA encoding a

human homolog of chicken MafF (hMafF) was isolated. This cDNA encodes an 18 kDa protein containing an extended basic leucine zipper (bZip) structure, but lacking a transactivation domain. hMafF mRNA and protein were detected in term myometrium and kidney, but not in nonpregnant myometrium or first-trimester myometrium [44]. Proteins containing a bZip structure interact with various families of transcription factors to activate or repress target gene expression. Ye et al used a novel human protein (GenBank AF289559), containing a coiled-coil domain of 48 amino acid residues with a typical heptad repeat of leucines, as bait for yeast two-hybrid screening and screened a human placental cDNA library. Interestingly, they isolated hMafF cDNA and named the bait protein they used as MafF interacting protein (MIP). MIP physically interacts with hMafF protein. Cotransfection of MIP and hMafF cDNA enhanced promoter activity containing a multiple US-2 element repeated sequence in HeLa cells and in yeast [45,46]. However, there is neither proof of MIP existence in human term myometrium, nor functional analysis of MIP and hMafF on the OTR promoter as yet. Small Maf proteins including MafF are considered to heterodimerize with Nrf (NFE2 related proteins) family transcription factors and modulating the activity of genes carrying antioxidant regulatory elements [47]. Some of them relate to a chemoprotective system against carcinogens. Murine MafF knockout mice did not reveal any novel phenotypes including reproductory dysfunction [48]. Therefore, the actual function of MafF on parturition should be further examined.

Epigenetic modulation of the OTR gene

In the human OTR gene, there is a long CpG island −140 bp to +2383 bp. Within this CpG island, the gene methylation level of the 405 bp fragment (MT2) was highest in liver compared to nonpregnant and term myometrium. A hepatocellular carcinoma cell line (HepG2) was cultured with 5-azacytidine to demethylate genomic DNA. HepG2 cells originally expressed minimal OTR mRNA and its expression level was upregulated after demethylation treatment. OTR-reporter gene plasmids were enzymatically methylated *in vitro* and transfected into HepG2 cells. When the plasmid contained the MT2 region, it showed the highest suppression of reporter activity. Therefore, methylation of the MT2 region has a significant effect on transcription of the OTR gene [49]. Recently, the relationship of OTR deficiency in the brain and autism has been extensively examined. There is a report that aberrant methylation of this MT2 region is observed in the DNA extracted from cortex of autistic patients, which might relate to the shortage of OTR expression in the patients' brains [50]. The OTR gene contains a large intron, and gene methylation status within this intron 3 may also regulate tissue specific transcriptional suppression [51]. Modification of the OTR gene by methylation may permit its tissue specific expression in the body.

Summary

There is plenty of information about OTR regulation; however, none of it explains direct and straightforward physiological regulation of OTR at parturition at the molecular level. Pharmacological experiments suggest that oxytocin-induced myometrial contractions might be modulated by contractile machinery rather than the quantity of OTR [52]. Pregnant mice deficient for the receptor prostaglandin F2α express lower levels of OTR. However, they successfully

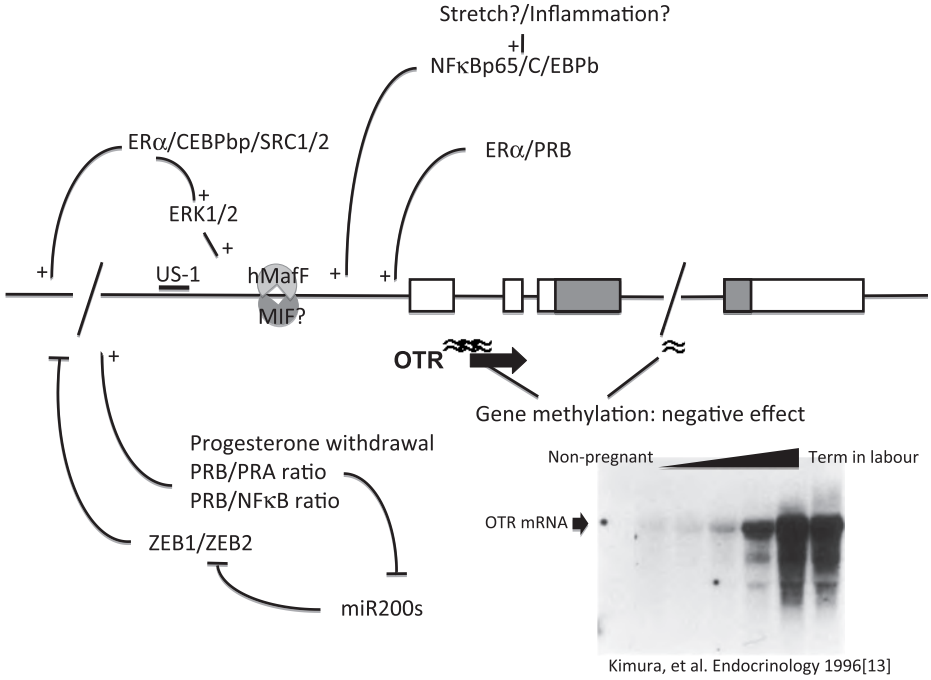

Fig. 1. Hypothesis for oxytocin receptor regulation. Oxytocin receptor is drastically upregulated during the course of pregnancy (see northern blotting of myometrial RNA). The mechanism of transcriptional regulation has been widely investigated, although not all studies are conclusive.

delivered their pups by continuous infusion of oxytocin at a higher dose [53]. These results might reflect the complex regulation of OTR-contraction mechanism *in vivo*. Oxytocin and OTR knockout mice delivered their pups without any problems [54−56], suggesting that the oxytocin-OTR system is not essential for parturition. These animal models revealed social behavior deficits, and recent research into the oxytocin-OTR system focuses on neuroscience, rather than obstetrics. Phenotypes of knockout model mice are often covered by redundancy. Indeed, it is reported that the genotype of OTR in humans may predict the length of the first stage of labor [57]. Drastic upregulation of OTR in myometrium at parturition suggests that molecular analysis of OTR regulation in myometrium may be a good probe for understanding parturition (Fig. 1). We expect that via investigation of molecular mechanism of OTR regulation, we could find the molecular target to cure preterm birth as well as diseases from abnormal uterine contractions.

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