Molecular regulation of the oxytocin receptor in peripheral organs

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Abstract

The oxytocin receptor belongs to the G-protein-coupled seven transmembrane receptor superfamily. Its main physiological role is regulating the contraction of uterine smooth muscle at parturition and the ejection of milk from the lactating breast. Oxytocin receptor expression is observed not only in the myometrium and mammary gland but also in the endometrium, decidua, ovary, testis, epididymis, vas deferens, thymus, heart and kidney, as well as in the brain. The expression profile shows a tissue-specific as well as a stage-specific pattern. The oxytocin receptor gene is a single-copy gene consisting of four exons and three introns, localized at 3p25–3p26·2 in the human chromosome. In transfection studies using a fusion construct containing the promoter region of the oxytocin receptor gene inserted in a reporter plasmid, neither proinflammatory cytokines nor oestrogen directly activate the gene. The nuclear fractions from up-regulated (term myometrium) and down-regulated (non-pregnant myometrium) tissues show differential patterns of protein binding to the 5′-flanking region, and a human homologue of chicken MafF has been cloned as a term-myometrium-specific oxytocin receptor modulator. The oxytocin receptor gene appears to be highly methylated. Methylation around intron 1 and in intron 3 might contribute to tissue-specific suppression of the gene. The oxytocin receptor is also regulated by desensitization, whose mechanism appears to involve loss of ligand-binding activity of the protein as well as suppression of the oxytocin receptor mRNA transcription. These findings taken together indicate that the oxytocin receptor is regulated in a very complicated manner, and the transcriptional regulatory elements critical for this regulation should be investigated further.

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Introduction

Oxytocin was the first peptide hormone whose primary structure was identified (du Vigneaud et al. 1953). Soon after its structure was identified, the bioactive peptide was chemically synthesized and clinically applied for induction and augmentation of labour. The first recognized physiological actions of oxytocin were stimulation of uterine contraction and milk ejection. In the 1980s, many studies showed that intracerebral infusion of oxytocin influences the sexual, maternal and social behaviour of experimental animals, suggesting a central regulatory role of oxytocin (Richard et al. 1991). The signalling of oxytocin is transduced to physiological actions via the oxytocin receptor (OTR). The OTR was first identified in the rat mammary gland using a pharmacological ligand-binding assay (Soloff & Swartz 1973). In the uterus, the first known target tissue of oxytocin, the level of
the receptor protein as detected in a ligand-binding assay increases dramatically at the time of parturition (Soloff et al. 1979, Fuchs et al. 1984). The molecular cloning of OTR (Kimura et al. 1992) not only revealed the primary amino acid sequence of OTR, but also provided a whole new set of tools and methods for analysing the distribution and regulation of oxytocin-dependent organs and functions. In this review, we focus on the topic of OTR regulation, mainly in peripheral organ systems.

The OTR gene and its transcriptional regulation

The expression of OTR has been precisely characterized not only in the uterine myometrium and mammary gland, but also in the endometrium, decidua, ovary, testis, epididymis, vas deferens, thymus, heart and kidney, as well as in the brain (reviewed by Kimura & Ivell 1999, Gimple & Fahrenholz 2001). Among these organs, the myometrium, mammary gland, endometrium, decidua, kidney and brain have been found to display dynamic regulation of OTR expression. Oxytocin even plays a pivotal role during cardiomyocyte differentiation through authentic OTR (Paquin et al. 2002). In order to elucidate the tissue-specific and stage-specific expression mechanisms, analysis of the OTR gene structure was necessary. The gene for OTR was found to be a single copy, mapped at 3p25–3p26·2 in the human genome (Inoue et al. 1994). Presently, the gene structures from several species, including the rat (Rozen et al. 1995), bovine (Bathgate et al. 1995), mouse (Kubota et al. 1996) and vole (Young et al. 1996) are available. The presence of OTR subtypes was suggested by ligand-binding experiments (Chan et al. 1993) but, to date, no subtypes have been identified by cross-species low-stringency genomic library screening.

Comparative studies of the promoter regions of homologous genes from several species often give clues about the important regulatory elements. Based on such comparative analyses as well as the clinical relationship between intrauterine infection and the onset of preterm birth, many investigators hypothesized that OTR transcription would be up-regulated by proinflammatory cytokines such as interleukin (IL)-1 and IL-6 via activation of the binding elements for nuclear factor (NF)-IL-6 or Stat3 (Inoue et al. 1994). In cultured uterine smooth muscle cells obtained at Caesarean section, IL-6 up-regulates OTR expression in a tyrosine kinase-dependent manner (Rauk et al. 2001). However, Schmid et al. (2001) reported that both IL-1β and IL-6 down-regulate the transcription of a reporter gene connected to the OTR gene promoter transfected into a human myometrial cell line (ULTR). They showed that the NF-IL-6 level appeared to be increased by IL-1β or IL-6 in ULTR cells, but that a promoter construct containing NF-IL-6 binding elements (−909/+108) was negatively regulated by IL-1β or IL-6 stimuli.

In the human mammary carcinosarcoma Hs578T cell line, OTR expression is up-regulated by serum treatment through a protein kinase C-dependent pathway. Dexamethasone shows a synergistic effect on serum-treated OTR up-regulation in this cell line (Coppard et al. 1999). Basal promoter activity is conferred by the 85 bp flanking the 5′ end of the human OTR gene. In this sequence, there is a consensus ets binding sequence. GABPα/β, which binds to the ets element, cooperates with c-fos/c-jun to activate OTR promoter transcription in Hs578T cells (Hoare et al. 1999). Although these transcription factors are important for OTR up-regulation in response to serum stimulation, it has not been determined whether they actually act in vivo under conditions of 100% serum. In rabbit amnion cells, in vitro treatment with forskolin and/or cortisol activates OTR gene transcription (Jeng et al. 1998). Protein kinase A, protein kinase C and nerve growth factor-dependent OTR up-regulation is also observed in MCF-7 and SK-N-SH cells transfected with the rat OTR gene promoter (Bale & Dorsa 1998). The physiological significance of these pathways in various settings (i.e. at parturition, during lactation, in the brain, etc.) should be elucidated further.

Oestrogen has been considered to be a key substrate for OTR regulation. Indeed, in vivo administration of oestrogen to castrated rats increases the number of OT-binding sites and the level of OTR mRNA in the myometrium and ventromedial nucleus of the brain. In the OTR 5′-upstream region, widely spaced 5′- and 3′-oestrogen responsive element (ERE) half sites are present. Such ERE half sites can act synergistically
in the chicken ovalbumin gene. However, they do not affect transcription of the OTR gene in cells co-transfected with estrogen receptor (ER) α cDNA under oestrogen stimulation (T Kimura, unpublished observations). Authentic palindromic ERE has been found about 4 kb upstream of the rat OTR gene. This sequence was found to activate reporter gene transcription only in the human breast cancer cell line (MCF-7) and only when the 3·3 kb fragment between ERE and the basal promoter of the rat OTR gene was truncated. In the wild-type gene construct, this ERE had no effect on the reporter transcription (Bale & Dorsa 1997). Vasudevan et al. (2001) indicated that 4·5 kb upstream of rat OTR promoter transcriptional activity is facilitated by ERα and inhibited by ERβ in the co-transfected neuroblastoma cell lines (CV-1 and SK-N-BE2C). In vitro translated ERα binds to the authentic ERE in the rat OTR gene. The authentic ERE in the far upstream of the rat OTR gene appears to be functional, at least in the neuron-derived cells. On the other hand, oestrogen administration to castrated rat up-regulated OTR mRNA expression in the uterus whereas it did not affect its expression in the mammary gland. Even in the identical endocrine circumstance in vivo, the regulation of the OTR gene is different depending on the tissue (Breton et al. 2001). In the human OTR gene, we could not find such a palindromic ERE in the area comparable to that of the rat gene (Y Mizumoto & T Kimura, unpublished observations). These facts suggest that the oestrogen effect on OTR transcription may be indirect rather than a direct oestrogen–ER–ERE–target gene effect at least in the peripheral organs. Indeed, although ERα is absolutely necessary for the induction of OTR in the brain by exogenous oestrogen administration, it is not necessary for basal OTR synthesis in ERα knock-out mice (Young et al. 1998). These observations also indicate that the effect of oestrogen on OTR regulation is not straightforward. Other interesting examples are provided by the tammar wallaby and the rat. Tammar wallaby has separated twin uteri, only one of which becomes gravid during pregnancy. The mesotocin receptor, which is the eutherian parologue of the OTR, is massively up-regulated immediately prior to parturition, but only in the gravid uterus. In the non-gravid uterus, the mesotocin receptor level is actually suppressed during the same time period below the basal level (Parry et al. 1997). In the rat, the mechanically stretched, non-gravid uterine horn shows equivalent OTR up-regulation to the gravid horn at parturition. On the other hand, the non-gravid, non-stretched horn shows no OTR up-regulation during labour of the gravid horn, even though they should be under the same endocrine conditions (Ou et al. 1998). Thus, the OTR up-regulation at parturition appears not to be regulated purely by circulating effectors such as oestrogens. Instead, local substrates or mechanical stretch produced by the fetus/placenta/deciduas might play pivotal roles in this setting.

Despite the vigorous efforts exerted in transcriptional regulatory studies, no transactivator has as yet been definitely identified (reviewed by Ivell et al. 2001). There are several factors that make investigations in this field difficult. Pregnant myometrial cells should be the best model to analyse, since it is not possible to induce ‘pregnancy’ in cultured cells. Transferring tissue samples obtained at Caesarean section to primary culture is possible; however, the cells lose their smooth muscle cell characteristics within a short period. We have tested several kinds of cell lines, but none of them expressed OTR at as high a level as the pregnant myometrium; therefore, at present no good in vitro cell model for massive up-regulation of OTR is available. Despite these unfavourable circumstances, our group tried to analyse DNA binding proteins that bind to the human OTR gene promoter region. We compared the specific DNA protein binding patterns of the nuclear extract purified from non-pregnant and term myometrium by electrophoretic mobility shift assay (differential display EMSA). We prepared 8 DNA fragments that together spanned nucleotides −637 to −1833 of the human OTR gene (+1 = transcription start site) and performed differential display EMSA with these labelled DNA fragments. Analysis of several shifted signals up-regulated in term myometrium allowed us to identify two protein-binding elements (US-1: −1745 to −1729 bp; US-2: −1433 to −1414 bp) by methylation interference footprinting. The concatemarized US-1 sequence showed weak enhancer activity in the SKN human leiomyosarcoma cell line. US-1 binding protein with a molecular weight of approximately 70 kDa was partially purified. However, we have not succeeded in achieving further purification of this protein or its expression cloning using the yeast
one-hybrid system with this element (Kimura et al. 1999a). For the other term myometrial nuclear protein binding element, US-2, we applied the yeast one-hybrid system and thereby cloned a homologue of chicken MafF, human (h) MafF. hMafF is a 17.8 kDa polypeptide possessing an NxxYAxxC motif and heptad repeats of a leucine zipper domain. This molecule which lacks a transactivating domain, belongs to the small Maf protein family (Kimura et al. 1999b). Indeed, co-transfection of a human OTR promoter-reporter plasmid containing the US-2 element and hMafF cDNA into MCF-7 cells does not result in any alteration in the basal reporter transcription activity. hMafF mRNA is up-regulated in term myometrium as well as in the kidney. Since hMafF contains a leucine zipper structure, it has the potential to form heterodimers with other leucine zipper transcription factors, and the possibility of such interactions should be elucidated further. Theoretically, many of the DNA-binding proteins expressed in term myometrium related to the OTR gene promoter could be characterized by these strategies. However, the characterization of DNA binding factors requires large amounts of nuclear proteins, and achieving this goal is likely to involve a lot of difficulty.

**Epigenetic regulation of OTR expression**

Several lines of unpublished observations suggest the epigenetic modification of the OTR gene. The first is that when an OTR gene promoter–reporter construct was transfected into cell lines such as HeLa cells, which express very low levels of OTR mRNA, the transcriptional activity was generally high, usually as much as ten times higher than the activity of an OTR promoter-less construct (T Kimura, R Bathgate & R Ivell, unpublished observations). The second is that when the knock-in construct loxP-exon2, 3 of the mouse OTR gene loxP-PgkNeo-loxP was introduced into mouse E14TG2a embryonic stem cells, the efficiency of homologous recombination was very low (from 2 x 10^3 transfected cells only two clones were obtained, which was less than 1/100 of the usual efficiency; Y Takayanagi, M Kawamata, T Kimura & K Nishimori, unpublished observations). These observations suggest that the OTR gene structure in the genomic chromatin may be highly modified. When the hepatocellular carcinoma HepG2 cell line is treated with a demethylating agent, 5-azacytidine, the amount of OTR mRNA in the cells is increased ninefold, although treatment with trichostatin A has no effect. In the human OTR gene region, there is a CpG-rich stretch from −140 to +2300 bp. Within this region, a stretch of approximately 400 bp (+176 to +581) is less methylated in OTR-expressing tissues (non-pregnant and term myometrium) than in non-expressing tissues (liver and peripheral mononuclear leukocytes). In vitro methylation of a reporter plasmid containing the CpG-rich stretch of the OTR upstream region suppresses the transcription activity, and deletion of the 400 bp fragment abrogates this suppression. This finding suggests that the methylation of this 400 bp region (+176 to +581) may be important for the tissue-specific suppression of OTR expression (Kusui et al. 2001). Within the large (>10 kb) third intron of the OTR gene, there is a 1.5 kb KpnI/EcoRI fragment which is less methylated in myometrial genomic DNA than in the DNA from peripheral blood leukocytes. This region includes a 20-mer element that can bind to nuclear proteins purified from myometrium. A Myb-binding element exists near this element, but nothing is known about the protein(s) that binds to this element (Mizumoto et al. 1997). The differential methylation pattern of the genomic DNA and differential nuclear protein-binding pattern suggest that this region may also be involved in transcriptional suppression. Moreover, knock-in mutant mice in which the loxP-PgkNeo-loxP sequence is inserted into the third intron of the mouse OTR gene show less than 10% of normal OTR expression in their myometrium at parturition and mammary glands even though they have an intact exon sequence (Y Takayanagi, M Kawamata, T Kimura & K Nishimori, unpublished observations). Taken together, these facts imply that intron 3 plays an important role in the transcriptional regulation of the OTR gene.

**Post-translational regulation of the OTR protein**

Oxytocin secretion is characterized by its pulsatile pattern. Continuous infusion of oxytocin has been widely used for the induction and augmentation of labour, and for the prevention of postpartum haemorrhage. It is effective in improving the
chances of vaginal delivery; however prolonged labour induction by continuous oxytocin infusion requires higher doses of oxytocin to stimulate uterine contraction and may result in postpartum atony and bleeding. These clinical observations suggest the existence of a desensitization mechanism for post-transcriptional regulation of the OTR, like those previously demonstrated for a variety of other G-protein-coupled receptors. Oxytocin-induced OTR desensitization in primary cultured human myometrial cells, which occurs within 12 h of continuous oxytocin stimulation, is concentration-dependent, and is characterized by a reduction in the number of oxytocin binding sites. However, the total amount of the OTR protein in the cell lysate as determined by Western blotting, as well as the amount of OTR protein on the cell surface detected by flow cytometry with anti-OTR monoclonal antibody, is unchanged after as compared with before oxytocin stimulation. The amount of OTR mRNA, in contrast, is down-regulated. These observations suggest that the OTR molecule is somehow modified such that it loses its oxytocin-binding activity, and that de novo translation of OTR protein would be reduced due to a decrease in the OTR mRNA level (Phaneuf et al. 1997). The OTR density as well as the OTR mRNA level was indeed sharply decreased in human myometrium obtained at Caesarean section after prolonged oxytocin-augmented or oxytocin-

**Figure 1** Complicated regulation of the OTR gene. Sex steroids are the most potent regulators of OTR expression; however there is no evidence for direct regulation, except for the rat OTR gene in the neuron-derived cell lines. Proinflammatory cytokines also show the opposite effect in *in vitro* experiments compared with the *in vivo* up-regulation observed at preterm birth due to infection. Oxytocin appears to down-regulate OTR transcription via an undefined pathway. According to the observation in the tammar wallaby, substrates derived from the feto-placental unit, which would determine the time of parturition, may have an effect on OTR expression in the uterus. Indeed, several DNA binding proteins (hMafF, GABPα/β, AP-1) bind to the OTR gene upstream, but their functions *in vivo* are unknown. Various physiological roles of oxytocin in central and peripheral organs appear to be mediated and regulated by OTR expression, and the mechanisms of their regulation, especially *in vivo*, should be elucidated further. ex, exon; E2, oestradiol; P4, progesterone; Me, methylation.
induced labour (Phaneuf et al. 2000), indicating that OTR desensitization actually occurs in vivo during labour.

Another pattern of desensitization has been reported for β-arrestin-dependent OTR internalization. Oakley et al. (2001) reported OTR internalization in transfected HEK-293 cells and argued that this internalization depends on agonist-dependent phosphorylation of the C-terminus of the receptor. They performed parallel experiments with the neurotensin-1 receptor and angiotensin II type I receptor. Mutation of putative phosphorylation sites in the C-terminus of the OTR disturbed its internalization as well as that of the other two receptors; however, phosphorylation of the mutant OTR was less disturbed than that of the other mutant receptors. Therefore, it is doubtful whether these C-terminal phosphorylation sites are critical for OTR internalization. Moreover, the kinetics of β-arrestin trafficking and OTR internalization were very fast (within 30 min) in HEK-293 cells. Thus, Oakley et al. (2001) might have been examining phenomena different from those occurring physiologically in the myometrium, and may instead have characterized a model for oxytocin as a neurotransmitter in the central nervous system.

**Conclusions**

In this review, we have focused mainly on investigations of OTR gene regulation in peripheral systems, especially on tissue-specific and time-specific OTR expression patterns. Although a wide variety of experimental strategies have been applied to this problem, no definitive regulatory mechanism has emerged even in the myometrium, in which the most drastic OTR up-regulation occurs (see Fig. 1). In the central nervous system, the oxytocin–OTR system may be involved in human pathological conditions such as anorexia nervosa (Demitrack et al. 1990), depression (Purba et al. 1996), obsessive-compulsive disorder (Leckman et al. 1994), or Prader-Willi syndrome (Swaab et al. 1995), as well as the physiology of sexual arousal (Bläicher et al. 1999). In some experimental animals, the oxytocin–OTR system is the most powerful factor in the induction of maternal behaviour. However, the central effect of oxytocin on behaviour shows striking species differences, probably caused by species differences in OTR distribution in the brain (Insel et al. 2001). This difference probably depends on the structure of the 5′-flanking region of the OTR gene, although no critical regulatory element has been identified there (Young et al. 1997). On the other hand, up-regulation of OTR in the myometrium prior to parturition occurs ubiquitously in mammals, and probably depends on common sequences in the OTR genes which have not been fully defined yet. In conclusion, various physiological roles of oxytocin in central and peripheral organs appear to be mediated and regulated by OTR expression, and the mechanisms of such regulation, especially in vivo, should be elucidated further.

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**References**


Purba JS, Hoedendijk WJG, Holman MA & Swaab DF 1996 Increased number of vasopressin- and oxytocin-expressing neurons in the paraventricular nucleus of the hypothalamus in depression. Archives in General Psychiatry 53:137–143.


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