The regulation of oxytocin and oxytocin receptor in human placenta according to gestational age

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Abstract

Oxytocin (OXT) is a peptide hormone that plays a central role in the regulation of parturition and lactation. OXT signaling is mediated by OXT receptor (OXTR), which shows species- and tissue-specific expressions and gene regulation. In the present study, we examined the synthesis of OXT and OXTR in human placenta tissue according to gestational age. A total of 48 placentas were divided into early preterm, late preterm and term groups depending on gestational age, and expression of OXT and OXTR was evaluated. First, OXT and OXTR mRNA and protein were detected in normal placenta tissue via Q-PCR, Dot-blot and Western blot assay. Both OXT and OXTR levels in normal placenta increased gradually in the late stage of pregnancy, suggesting that local OXT may play a critical role in the function of the placenta. To determine the regulatory mechanism of OXT, placental BeWo cells were administrated estrogen (E_2) or progesterone (P_4), and expression of OXT and OXTR was tested. The mRNA and protein levels of OXT and OXTR were upregulated by E_2 but blocked by co-treatment with P_4 . In order to confirm the estrogen receptor (ESR)-mediated signaling, we administrated ESR antagonists together with E_2 to BeWo cells. As a result, both OXT and OXTR were significantly altered by ESR1 antagonist (MPP) while moderately regulated by ESR2 antagonist (PHTPP). These results suggest that OXT and OXTR are controlled mainly by E_2 in the placenta via ESR1 and thus may play physiological functions in the human placenta during the late stage of pregnancy.

Key Words

- ▶ placenta
- ▶ oxytocin
- ▶ oxytocin receptor
- ▶ estrogen
- ▶ estrogen receptor

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Introduction

The placenta is a transient tissue that performs many functions for the maintenance of pregnancy. In humans, the specific structure of the placenta is apparent as

early as 21 weeks of pregnancy (Benirschke *et al.* 1998). During pregnancy, the placenta is uniquely organized for exchange of nutrients, oxygen, hormones, antibodies and

waste products between the mother and fetus and may transfer valuable information for the pregnancy (Sood *et al.* 2006). These functions are realized by bringing maternal and fetal blood into close apposition (Enders & Blankenship 1999).

The placenta also performs hormonal functions in both maternal and fetal circulation to affect pregnancy, metabolism, fetal growth and parturition (Gude *et al.* 2004). Placental hormones such as human chorionic gonadotropin (hCG), progesterone (P_4) and estradiol (E_2) are important for pregnancy maintenance. Detection of hCG in the urine and blood is commonly used as a pregnancy test, as it is only produced during pregnancy (Cole 2012). P_4 is also a crucial hormone for gestational maintenance. From 6–8 weeks of gestation until the end of pregnancy, the placenta gradually becomes the main source of P_4 (Tuckey 2005). P_4 , which has multiple functions throughout the gestational period, acts on the endometrium to promote uterine quiescence and inhibits myometrium contractility. In other organs, P_4 prepares mammary glands for lactation by increasing proliferation of the mammalian epithelium, while at the same time, preventing lactation until labor by inhibiting prolactin secretion (Pang & Hartmann 2007).

During the first weeks of gestation, E_2 is mainly produced by the corpus luteum, after which the placenta synthesizes this steroid. The effects of E_2 are typically controlled by the activation of nuclear estrogen receptors, estrogen receptor 1 (ESR1) and estrogen receptor 2 (ESR2). In the placenta, ESR1 expression is normally confined to the cytotrophoblast, whereas ESR2 is more highly expressed in the syncytiotrophoblast (Bukovsky *et al.* 2003, Bechi *et al.* 2006). The concentration of E_2 increases by as much as 80-fold during pregnancy (Bukovsky *et al.* 2003). The role of E_2 in gestation is extremely varied, from promoting growth of the endometrium and preparing the uterus for implantation to bringing about maternal cardiovascular adaptations (Magness & Rosenfeld 1989, Burton *et al.* 1999, Albrecht *et al.* 2000, King & Critchley 2010). E_2 also causes angiogenesis and vasodilatation in the placental tissue, which indicates a role in the regulation of human utero-placental blood flow (Costa 2016).

Oxytocin (OXT) is a nanopeptide hormone that plays many roles in physiological processes, including those related to reproduction such as maternal behavior, labor, lactation and ejaculation (Bethlehem *et al.* 2013). OXT is synthesized by magnocellular neurons of supraoptic and paraventricular nuclei of the hypothalamus, whose axons terminate within the posterior lobe of the pituitary gland

(Arrowsmith & Wray 2014). OXT is also synthesized by peripheral tissues, including the uterus, corpus luteum, amnion and placenta (Arrowsmith & Wray 2014). OXT signaling is mediated by oxytocin receptor (OXTR), which is involved in the rhodopsin-type G protein-coupled receptor family (Sladek & Song 2012). OXTR is coupled to phospholipase C, which commands the synthesis of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate. Consequently, after the activation of OXTR, inositol triphosphate causes an increase in Ca^{2+} influx from both intracellular and extracellular stores, whereas DAG prompts the activation of protein kinase type C, which phosphorylates target proteins (Koehbach *et al.* 2013). Although the physiological functions and signaling pathway of OXT have been established in other organs such as the brain, uterus and mammalian gland, there are limited studies available in the placenta. Furthermore, although OXT synthesis was tested in the rat placenta, OXT signaling has never been examined in the human placenta, which has different morphogenesis and endocrine function from rat (Lefebvre *et al.* 1992).

In this study, we examined the local synthesis of OXT and expression of OXTR in the human placenta according to gestational age. Regulation of OXT and OXTR by sex steroid hormones E_2 and P_4 was also explored in human placental BeWo cells.

Materials and methods

Tissue and plasma collection and processing

This study was approved by the Institutional Review Board of the Pusan National University Hospital Clinical Trial Center (H-1302-005-015), and all participants gave written informed consent. Placental tissue and plasma samples were obtained from pregnant women who met the following inclusion criteria: (1) singleton pregnancy, (2) normal pregnancy at the time of sample collection and (3) healthy women with no preexisting clinical conditions such as diabetes, hypertension or autoimmune disease. The placental samples were divided into early preterm ($n=10$, 2 were from vaginal delivery and 8 were from C-section), late preterm ($n=18$, 6 were from vaginal delivery and 12 were from C-section) and term ($n=20$, 3 were from vaginal delivery and 17 were from C-section) groups after onset of labor as indicated in the previous study (Kim *et al.* 2016). The early preterm period was determined to be 22–29 weeks of gestation, which is clinically important since preterm delivery frequently occurs during this period, whereas the late preterm period was determined

Table 1 Clinical characteristics of pregnancies.

	Early preterm (n=10)	Late preterm (n=18)	Term (n=20)
Gestational age (weeks)	27.5±4.7	34.2±8.2	38.4±1.1
Birth weight (g)	887.0±312.5	2316.1±570.7	3167.0±268.8
Systolic BP (mmHg)	111.0±11.0	107.8±8.1	108.0±7.7
Diastolic BP (mmHg)	73.0±8.2	67.8±8.1	68.5±7.5
Parity	0.6±1.0	0.7±0.8	0.7±0.6
Gravidity	2.4±1.3	2.5±1.5	2.2±1.0
BMI (kg/m ²)	23.1±2.6	25.7±3.4	25.0±1.6

to be 30–36 weeks of gestation. The term placenta group was collected from a gestational age of 37–40 weeks. The plasma samples were collected at the 1st stage of labor and stored until the experiment. The clinical characteristics of sample groups are shown in Table 1.

Cell culture and treatments

The BeWo human choriocarcinoma-derived cell line was cultured in Dulbecco's modified Eagle medium (DMEM, Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS, Hyclone) and 1% streptomycin/penicillin (Welgene Inc., Seoul, Korea) and grown in 5% CO₂ at 37°C. In order to mitigate the effect of endogenous steroids, cells were cultured in phenol red-free medium containing 5% (v/v) charcoal dextran-stripped serum (DMEM-5%CD, Hyclone) for 2 days. The experimental treatment (ethanol as control; 10^{−7} and 10^{−5} M of E₂ and P₄, respectively, alone or in combination with equivalent concentrations of each compound) was performed in triplicate wells. The experiments were repeated at least three times independently. Cells were harvested 24 h after treatment to measure mRNA and protein levels.

To examine the signaling of E₂ in combination with certain receptors, cells were treated with 10^{−6} M of selective ESR1 and ESR2 antagonists: 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP, Tocris Bioscience, MO, USA) and 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo(1,5-a)pyrimidin-3-yl]phenol (PHTPP, Tocris Bioscience), respectively, in DMEM for 24 h.

Measure of blood OXT concentration

Blood was collected from each volunteer and collected in a plastic tube under aseptic conditions with EDTA as an anti-coagulant and then centrifuged in order to separate plasma. Plasma was stored at −70°C, and the sample was slowly thawed at room temperature before the experiment. Plasma OXT concentrations were measured

using an oxytocin-ELISA kit (ADI-900-153A-0001; Enzo Life Sciences, NY, USA) following the manufacturer's protocol. The ELISA kit is highly sensitive (minimal detection levels < 11.7 pg/mL OXT) and specific. The inter- and intra-assay coefficients of variation were < 10% and 11.9% for OXT.

Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA concentration was measured using a spectrophotometer. First-strand complement DNA (cDNA) was synthesized by reverse transcription from 3 µg of total RNA using moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen Co.) and random primers (9-mers; TaKaRa Bio Inc., Otsu, Shiga, Japan). Quantitative real-time PCR (Q-PCR) was performed with cDNA template (2 µL) and 2×Power SYBR Green (6 µL; TOYOBO Co., Osaka, Japan)-containing specific primers. Primer sequences for β-actin (control gene), OXT, OXTR and placental leucine aminopeptidase (P-LAP) are shown in Table 2. Q-PCR was carried out for 40 cycles using the following parameters: denaturation at 95°C for 15 s, followed by annealing and extension at 70°C for 60 s. Fluorescence intensity was measured at the end of the extension phase of each cycle. The threshold value for the fluorescence intensity of all samples was set manually. The reaction cycle at which PCR products exceeded this fluorescence intensity threshold during the exponential phase of PCR amplification was considered to be the threshold cycle (CT). Quantification was performed by comparing CT values at constant fluorescence intensity. The amount of transcript is inversely related to the observed CT, and for every 2-fold dilution of the transcript, the CT is expected to increase by one increment. Relative expression (R) was calculated using the equation $R = 2^{-(\Delta CT \text{ sample} - \Delta CT \text{ control})}$. To identify a normalized arbitrary value for each gene, each value obtained was normalized to the expression

Table 2 Primer sequences for real-time PCR analyses.

Gene name	Primer	Sequence (5'–3')	Fragment (bp)
β-actin	Forward	GGACTTCGAGCAAGAGATGG	234
	Reverse	AGCACTGTGTTGGCGTACAG	
OXT	Forward	GCTGCCAGGAGGAGAACTAC	178
	Reverse	GTGTTCGGAGCCATCAAGTT	
OXTR	Forward	TTCTTCGTCAGATGTGGAG	153
	Reverse	GGAGGAGTTGCTCTTTTTCG	

of β-actin, a ubiquitous housekeeping gene for placenta tissue. β-actin is one of the cytoskeletal actins that are involved in cell motility, structure and integrity. β-actin is an attractive candidate for reference coamplification because it exhibits only minor intraindividual kinetic changes and is not primarily affected by any human disease (Kreuzer *et al.* 1999).

Western blotting analysis

Protein samples were extracted with Pro-prep solution (iNtRON Biotechnology, Seoul, Korea) following the manufacturer's protocol. Cytosolic proteins (25 µg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Daeillab Service Co., Seoul, Korea). The membranes were then blocked for 2 h with 5% skim milk (Difco, Sparks, MD, USA) in Tris-buffered saline (TBS) with 0.05% Tween20 (TBS-T). After blocking, membranes were incubated with anti-OXTR goat (SC-8102, Santa Cruz Biotechnology, diluted 1:500) overnight, followed by HRP-conjugated anti-goat secondary antibody (SC-2020, Santa Cruz Biotechnology, diluted 1:2000) in 5% skim milk with TBS-T for 1 h. Luminol reagent (Bio-Rad) was used to visualize antibody binding. Each blot was then stripped by incubation with 2% SDS and 100 mM mercaptoethanol in 62.5 mM Tris-HCl (pH 6.8) for 30 min at 50–60°C. The membranes were subsequently probed with antibody against β-actin (#4967, Cell Signaling Technology, diluted 1:2000) as an internal control. The antibodies for OXTR (Young *et al.* 2014) and β-actin (Li *et al.* 2013) were selected based on previous publication. The blots were scanned using Gel Doc 1000, version 1.5 (Bio-Rad), and band intensities were normalized to β-actin levels.

Dot-blotting analysis

Cytosolic proteins (0.5 µg) were transferred onto a nitrocellulose membrane by using a Slot Blot kit

(Pharmacia Biotech, CA, USA). The membranes were then blocked for 30 min with 5% skim milk with TBS-T. After blocking, membrane was incubated with anti-OXT mouse (MAB5296, Merck Millipore, diluted 1:5000) for 1 h at room temperature. The antibody for OXT was chosen based on the previous report (Kohno *et al.* 2008). After being washed three times with TBS-T, the membrane was incubated with HRP-conjugated anti-mouse secondary antibody (SC-2005, Santa Cruz Biotechnology, diluted 1:2000) in 5% skim milk with TBS-T for 1 h. OXT proteins were then normalized with β-actin, visualized and detected by same methods with Western blotting.

Immunohistochemical staining

Placental tissues were fixed with 10% formalin and embedded in paraffin. Sections (4-µm thick) were then cut, deparaffinized in xylene and hydrated in descending grades of ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (H₂O₂) in methanol. After eliminating non-specific reactions by incubation with 2% bovine serum albumin (BSA) for 20 min, sections were incubated at room temperature for 2 h with antibodies against OXT (1:50) or OXTR (1:200), which were same as used for Western/dot-blot analysis. The sections were washed with TBS-T, followed by incubation with second antibody for 1 h at 37°C. Detection of primary antibody was performed with a Polink-2 Plus HRP DAB kit (GBI Labs, Mukilteo, WA, USA). Images of tissue were captured at 40× using a model BX50F-3 optical microscope (Olympus, Tokyo, Japan).

Data analysis

Results are presented as the mean ± standard deviation (s.d.). Data were analyzed by *t*-test using Sigma Plot 10.0 (Systat Software, Inc, San Jose, Calif). *P* values <0.05 were considered statistically significant.

Figure 1
Plasma OXT concentration according to gestational age. Plasma OXT concentration (pg/mL) was measured and represented.

Results

Plasma oxytocin concentration and its metabolic enzyme according to gestational age

For the initial experiment, plasma OXT concentration during gestation was examined. The plasma samples were

acquired from 23 normal pregnant women, and OXT levels were measured from gestational age between 22 and 40 weeks. The concentration of OXT ranged from 80 to 2324 pg/mL with a median value of 918 pg/mL. OXT plasma concentrations increased gradually from 22 week until the late stage of pregnancy (Fig. 1). The average values of OXT concentration were 607, 941 and 1.189 pg/mL in early preterm, late preterm and term periods, respectively. Expression of the metabolic enzyme of OXT in the placenta, *P-LAP*, showed no significant difference during gestation (data not shown).

Expression of OXT and OXTR in placenta according to gestational age

For the next experiment, we measured mRNA expression levels of OXT and OXTR in the placenta by Q-PCR. As shown in Fig. 2A and B, mRNA levels of both OXT and OXTR significantly increased in the term placenta against early preterm placenta by 3.3- and 4.5-fold, respectively, which were gestational age dependent. Protein levels of OXT and OXTR were also analyzed by Dot-blot and Western blot assays. We performed Dot-blot assay rather than Western blot to detect OXT protein due to its low molecular weight. The results show that OXT and OXTR

Figure 2

The mRNA and protein levels of OXT and OXTR in the human placenta according to gestational age. Total mRNA and proteins were harvested from the human placenta after the onset of labor. Transcriptional levels of OXT (A) and OXTR (B) were analyzed by Q-PCR depending on gestational age. Proteins were processed for Dot-blot assay and Western blot assay and quantified for graphs, and the representative samples are shown in (C) and (D). The values for OXT (E) and OXTR (F) are represented as schematic graphs. Total mRNA and protein expression levels were normalized to that of β -actin. Data were expressed as the mean $\pm$ s.d. * $P < 0.05$ compared to the early preterm group.

protein levels gradually increased until the late stage of pregnancy (Fig. 2C and D). These results were consistent with those of mRNA. The bands of β -actin were used as an internal standard in both the Dot-blot and Western blot to normalize the data.

Immunohistochemical analysis of OXT and OXTR in the human placenta

To further explore the localization of OXT and OXTR proteins in placental tissues, we performed immunostaining for OXT and OXTR. The placenta is basically composed of cytotrophoblasts and syncytiotrophoblasts. As shown in Fig. 3A, OXT was detected in both cytotrophoblasts and syncytiotrophoblasts, whereas OXTR signals were dominantly localized to syncytiotrophoblasts than cytotrophoblasts.

The regulation of OXT and OXTR in placental BeWo cells

In order to examine the regulation of OXT and OXTR gene expression, human choriocarcinoma-derived BeWo cells were employed. The two main sex steroid hormones, E_2 and P_4 , were administrated to BeWo cells at concentrations of 10^{-7} M and 10^{-5} M. The mRNA expression levels of OXT and OXTR were upregulated by 3.3- and 4.5-fold, respectively, whereas co-treatment of P_4 reduced the stimulation to the control levels (Fig. 4). Protein analysis of OXT by dot-blotting showed strong intensity possibly due to background signals from non-specific binding of total proteins (data not shown). Single treatment with P_4 did not significantly modulate the expression of genes. Since the actions of E_2 are classically mediated by its cognitive receptors, ESR1 and ESR2, inhibitors of ESR isoforms were further tested. BeWo cells were pretreated with MPP (ESR1 antagonist) or PHTPP (ESR2 antagonist) before E_2 , and expression levels of OXT and OXTR were examined (Fig. 5). In the results, effects of E_2 on OXT (Fig. 5A) and OXTR (Fig. 5B) were completely blocked by MPP, whereas PHTPP moderately reduced the increase.

Discussion

OXT is normally produced in the hypothalamus and stored in the posterior pituitary gland. However, OXT is also synthesized by peripheral tissues, including the corpus luteum, uterus lining, testes and amnion. It has also been reported that OXT is produced and secreted from placental tissue (Sakai *et al.* 1993). Although a

Figure 3 Immunostaining of OXT and OXTR in human placental tissue. To examine localization of each protein, tissues were stained with specific antibodies for OXT (A) and OXTR (B). A full color version of this figure is available at <http://dx.doi.org/10.1530/JME-16-0223>.

high concentration of OXT is associated with labor and regulation of uterine contractions, this action is only limited at the term period (Sugimoto *et al.* 1997). However, the physiological function of OXT during early- to mid-stage of pregnancy is not understood.

In this study, we examined the local production of OXT and its regulation in human placenta. It is known that OXT levels in the blood increase during pregnancy (Thornton *et al.* 1992, Veiga *et al.* 2015), which was also

Figure 4

Regulating *OXT* and *OXTR* expression levels by *E₂* in BeWo cells. After treatment of BeWo cells with *E₂*, *P₄*, and *E₂* + *P₄*, mRNA and proteins were harvested. Transcriptional levels of *OXT* (A) and *OXTR* (B) were analyzed by Q-PCR. Translational levels of *OXTR* (C) were analyzed by Western blot assay. Total mRNA and protein expression levels were normalized to that of β-actin. Data were expressed as the mean ± s.d. **P* < 0.05 compared to the control group.

confirmed in our ELISA results. Peripheral existence of *OXT* has been confirmed in several tissues. In 1993, a cDNA clone of *OXT* was separated from rat and human uterine tissues and verified by Northern blot analysis (Lefebvre *et al.* 1993, Sakai *et al.* 1993). Although many studies have reported the synthesis of *OXT* in the uterus, there have been few studies regarding *OXT* in the placenta. In this study, transcriptional and translational levels of

OXT and *OXTR* in the placenta were increased at late stage of gestation. Because the endogenous *OXT* can be metabolized by the P-LAP enzyme in the placental tissue, we analyzed the expression of P-LAP. In the results, the expression levels of P-LAP were not significantly altered during gestational stage, suggesting that the amount of *OXT* is not regulated by P-LAP in the placenta. Previously, it was reported that *OXT* mRNA levels in the human

Figure 5

Effects of ESR antagonists on *E₂*-induced *OXT* and *OXTR* expression. After treatment of BeWo cells with *E₂* alone or ESR antagonists, mRNA and proteins were harvested. The transcriptional levels of *OXT* (A) and *OXTR* (B) were analyzed by Q-PCR. Translational levels of *OXTR* (C) were analyzed by Western blot assay. Total mRNA and protein expression levels were normalized to that of β-actin. Data were expressed as the mean ± s.d. **P* < 0.05 compared to the *E₂*-treated group.

chorio-decidua greatly increased at the start of labor (Nakazawa *et al.* 1984).

It has been well known that E₂ and P₄ are high in the plasma at late pregnancy (Johansson 1969, Loriaux *et al.* 1972). Since the expressions of OXT and OXTR were enhanced during the late stage of pregnancy, we hypothesized that E₂ and/or P₄ might be potent regulatory factors for OXT and OXTR expression. To prove this, placental BeWo cells were treated with E₂ and/or P₄, and expression of OXT and OXTR was tested. In the results, OXT and OXTR mRNA and protein levels were upregulated by E₂ but not by P₄. Augmentation of OXT by E₂ was also detected in the uterus, ovaries and neurohypophysis previously (Amico *et al.* 1981, Shukovski *et al.* 1990). Chibbar and coworkers demonstrated that OXT is synthesized within human amnion, chorion and decidua during late gestation and the expression of OXT in chorio-decidua was stimulated by E₂ (Chibbar *et al.* 1995). For the next experiment, we verified the involvement of ESR isoforms in the regulation of OXT and OXTR. When BeWo cells were pretreated with MPP and PHTPP before E₂, the ESR1-specific inhibitor, MPP, completely blocked the regulation of both OXT and OXTR, suggesting that the effect of E₂ was dominantly mediated by ESR1. Involvement of ESR in the regulation of uterine OXT was also reported previously (Kimura *et al.* 1996), and the author demonstrated that OXT regulation by E₂ is mediated by ESR1, which is similar with our data.

In conclusion, our results indicate that OXT and OXTR were highly expressed in the placenta during the late stage of pregnancy compared with early preterm. The local production of OXT and OXTR in the placenta was regulated by E₂ but not by P₄ via mainly ESR1, and thus, may play physiological functions in the human placenta during the late stage of pregnancy.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

B S An and S C Kim initiated and designed the study and played a role in recruitment of patients and sample collection. J E Lee contributed toward acquisition of data. B S An, J E Lee, S C Kim, S S Kang, H S Yang

and S S Kim were involved in the analysis and interpretation of the data. J E Lee drafted the manuscript. All authors contributed to the revision of the manuscript and approval of the final draft.

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