Characterization of Oxytocin Receptor Expression and Distribution in the Pregnant Sheep Uterus*

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ABSTRACT

At the end of pregnancy, the myometrium becomes extremely sensitive to oxytocin (OT) as a result of a dramatic increase in the number of OT receptors (OTR), indicating an important role for OTR in the process of labor. There are no studies in sheep in which the physical properties and histological distribution of OTR are evaluated in relation to parturition. Also, no studies have been performed in any species to simultaneously examine the distribution of OTR at the messenger RNA (mRNA) as well as the protein levels in the same tissues and correlate those changes with the patterns of myometrial activity that occur at labor. In the present studies, we have used a polyclonal anti-OTR antibody and Western blot analysis to determine the apparent molecular mass of ovine OTR in late pregnant sheep myometrium and endometrium. We also examined the distribution of OTR mRNA and protein expression in the intact myometrium and endometrium and in individual cultured cells using in situ hybridization and immunocytochemistry. The expression of OTR and its mRNA has been correlated with the patterns of activity observed in the pregnant sheep myometrium. Western blot analysis of myometrial and endometrial extracts revealed a major form of OTR with an approximate molecular mass of 66 kDa. Both immunocytochemistry and in situ hybridization localized OTR and its mRNA in myometrial and glandular cells of the endometrium. Increased OTR and its mRNA expression in the myometrium and endometrium were correlated with the occurrence of myometrial contractions. OTR was also demonstrated by immunocytochemistry in the smooth muscle of myometrial blood vessels.

Localisation of OTR and its mRNA in pregnant sheep myometrium and endometrium is consistent with the hypothesis that OTR plays an important role in regulating myometrial contractility. Positive staining of OTR in endometrial glandular cells supports the view that OT is involved in PG production by the endometrium in late pregnancy. Increased expression of OTR and its mRNA in the myometrium further indicates that changes in tissue OTR play a significant role in the mechanism of parturition. Increased expression of OTR and its mRNA in endometrium may relate to the role of OT in regulating PG production by the endometrium during labor. (Endocrinology 1997; 137: 722–728, 1996)

OXYTOCIN (OT) plays a major role in the increase in myometrial contractility observed during labor at term. OT receptor (OTR) number, as measured by binding assays, increases in late pregnancy and during labor in both the myometrium and endometrium (1–4). In addition, the level of OTR messenger RNA (mRNA) measured by Northern blot analysis increases dramatically during labor in the pregnant sheep myometrium and endometrium (5). OT may exert its action on endometrial and myometrial cells as a result of changes in both the number of OTRs in the target cells and the concentration of OT in the circulation. Evaluation of the regional distribution of OTR is critical to analysis of the role of OTR in the regulation of the function of the various tissues of the uterus. Although changes in OT-specific binding sites have been extensively studied using ligand binding assays, the assessment and characterization of the cells that produce and express the OTR have been limited by the lack of a specific and sensitive anti-OTR antibody. Recently, the OTR gene has been characterized, and distribution of OTR mRNA expression has been examined in histological sections of human endometrium by in situ hybridization (6). However, there have been no studies that have correlated OTR gene expression with distribution of OTR protein in relation to the well characterized changes in myometrial activity that occur during labor in any species. The sheep has been extensively investigated in late gestation to evaluate the electrical and mechanical changes that take place at the end of pregnancy. However, neither the physical properties of the OTR nor its synthesis and distribution in the pregnant sheep myometrium and endometrium have been described.

Recently, an OTR antibody has been generated against the third intracellular loop of the rat OTR sequence (7). In the present studies, we examined the cellular distribution of OTR mRNA in relation to its protein expression in both the pregnant sheep myometrium and endometrium. We also evaluated the cellular localization of OTR in endometrial and myometrial cells cultured in vitro. Finally, we determined the apparent molecular mass of ovine OTR in both the myometrium and endometrium by Western blot using a polyclonal anti-OTR antibody and correlated the changes in OTR and its mRNA with precisely evaluated recording of the changes in myometrial contractility that occur during labor.
Materials and Methods

Care of animals and tissue collection

Fifteen pregnant Rambouillet-Dorset ewes bred only once and carrying fetuses of known gestational age were used for this study. Experimental procedures were approved by the Cornell University institutional animal care and use committee. The Cornell facilities are approved by the American Association for the Accreditation of Laboratory Animal Care. Ewes from which tissues were obtained during labor were instrumented at 120 days gestation (dGA) with electromyogram (EMG) leads in the myometrium and fetal and maternal cardiac arterial and jugular venous catheters (8). Labor was defined as having occurred when the EMG record showed a clear switch from myometrial contractions to contractions followed by contraction activity for at least 5 h (9).

To examine the effect of labor on OTR and its mRNA, tissues were obtained from ewes during labor induced by the infusion of betamethasone (Celestone phosphate, Schering, Bloomfield, IL; n = 6) or dexamethasone (Azium, Schering; n = 3; beginning at 127 dGA). Dexamethasone or betamethasone (total dosage, 0.5 mg) was administered iv into the fetal jugular vein over a period of 48 h in saline vehicle at a rate of 0.25 ml/h. Pregnant ewes were selectively necropsied 3 days after the infusion period or earlier if they went into labor due to glucocorticoid administration. Fetuses from contemporary control ewes (n = 6) at the same gestational age received equivalent volumes of physiological saline vehicle and were not in labor, as judged from myometrial EMG patterns. All tissues were obtained under halothane general anesthesia. Immediately upon collection, tissues (myometrium and endometrium) were treated in one of the following ways. One portion was placed in sterile Ca2+- and Mg2+-free HBSS (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS (Life Technologies, Grand Island, NY) for postmortem contractions. A second portion was frozen in precooled isopentane for in situ hybridization. A third portion was rapidly frozen in liquid nitrogen for extraction of protein and RNA. Frozen tissues were stored at -80 C before use. A fourth portion of tissues was fixed in 4% paraformaldehyde at room temperature for 24 h before being processed and embedded in paraffin for evaluation by immunocytochemistry (10).

Total RNA preparation and Northern blot analysis

Total RNA was prepared from individual tissues as previously described in detail (11). Briefly, total RNA was isolated from frozen tissue by homogenization in 4.2 M guanidinium thiocyanate solution. RNA was precipitated through a 5 M ammonium chloride and ethanol. The RNA purity and recovery of each tissue were determined by UV spectrophotometry (260 and 280 nm). There were no differences in the yield of RNA per mg tissue over the gestational ages studied or between ewes in labor and ewes not in labor. Purified RNA was resuspended in 1 mM EDTA and stored at -80 C.

Samples of total RNA (40 lgi/ml) from each tissue were denatured in 17.4% (vol/vol) formaldehyde, 50% (vol/vol) formamide, 20 mM MOPS [3-(N-morpholinolopropanesulfonic acid], 5 mM sodium acetate, and 1.0 EDTA, pH 7.0, for 5 min at 65 C and separated on a 1% (wt/vol) agarose-0.66 M formaldehyde gel. Ethidium bromide-stained ribosomal RNA (rRNA) bands were visualized (UV) to insure that RNA degradation had not occurred and an equal amount of RNA had been loaded into each lane. After electrophoresis, RNA was transferred to a nylon membrane (GeneScreen, New England Nuclear-DuPont, Wilmington, DE) by capillary blotting for 24 h in 10 x SSC (1 x SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and cross-linked by UV irradiation (Stratolinker, Stratagene, La Jolla, CA). The completeness and uniformity of transfer were assessed by determining the transfer of 28S and 18S RNA from the gel. Membranes were prehybridized at 42 C for 5 h in hybridization solution (50% [vol/vol] deionized formamide, 50% sodium phosphate, 0.8 M NaCl, 2% [wt/vol] SDS, 100 lgi salmon sperm DNA/ml, 20 lgi transfer RNA/ml, and 1 X Denhardt's solution (1 X = 1% solution of tRNA, Ficoll, and polyvinylpyrrolidone).

A 131-bp complementary DNA (cDNA) probe encoding part of the sheep endometrial OTR, which was generated by PCR, was kindly made available to us by Dr. Flint (12) and labeled with [32P]deoxy-CTP using the random priming kit (New England Nuclear-DuPont) to a specific activity of approximately 1 x 109 cpm//lg. Labeled rDNA was used as a final concentration of 1 x 106 cpm specific probe/ml hybridization solution.

Hybridization was carried out at 42 C for 20 h. Membranes were washed sequentially in 2 x SSC at room temperature for 10 min, and in 0.5 x SSC with 0.1% SDS at 65 C for 10 min. Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) was exposed to the membrane with intensifying screens at -80 C. Exposure durations were varied to achieve hybridization signals within the limited linear range required for densitometry.

Membranes were stripped of OTR probe by boiling in 0.1 X SSC with 0.1% (vol/vol) SDS for 30 min and rehybrized with a 32P-labeled 18S cDNA probe (kindly provided by Dr. A. Bemdtson, Cornell University) to normalize OTR mRNA levels. Autoradiographed signals were quantified by laser densitometry.

In situ hybridization

In situ hybridization was performed as described previously (10). Frozen sections (7 lgi) of pregnant sheep uterus were cut and thaw-mounted onto poly-L-lysine-coated slides. Tissue sections were fixed in 4% paraformaldehyde in 10 mm PBS for 10 min at room temperature and then washed in PBS (three times for 5 min each time). The sections were prehybridized at 42 C for 1 h in prehybridization buffer containing 50% formamide, 5 x SSPE [1 X SSPE is 0.18 M NaCl, 10 mM NaH2PO4 (pH 7.4) and 1.0 EDTA], 0.1% (wt/vol) SDS, 0.1% (vol/vol) Denhardt's solution, 200 lgi denatured salmon testis DNA/ml, and 200 lgi transfer RNA/ml. Hybridization was carried out for 18 h at 42 C in hybridization buffer [prehybridization buffer plus 4% (vol/vol) dextran sulfate] containing 1.5 x 106 cpm 32P-labeled ovine OTR cDNA probe/section. The probe used for in situ hybridization was the same probe as that used for Northern analysis. After hybridization, the sections were rinsed at room temperature for 2 h in 2 x SSC, 2 h in 1 x SSC, 1 h in 0.5 x SSC, and finally 1 h in 0.5 x SSC at 37 C. The sections were then dehydrated by passing through an alcohol series containing 50% ammonium acetate and coated with liquid photographic emulsion (Kodak, NTB2) after 3 weeks of exposure, the sections were developed and stained with hematoxylin.

Controls

Serial sections were treated with pancreatic ribonuclease A (20 lgi/ml) for 30 min at room temperature before hybridization procedures. After enzyme pretreatment, the sections were rinsed in three changes of 2 x SSC (5 min each) and hybridized with the labeled probe, as described above.

Cell dispersion

Myometrium and endometrium were washed twice in HBSS without FBS supplement. The endometrium was gently removed with fine forceps and scissors, and the separated myometrium and endometrium were rinsed in HBSS and cut into 10-mm pieces. The myometrium and endometrium were incubated for 1 h in a water bath at 37 C in 10 ml HBSS containing 300 IU/ml collagenase (type I-A, Sigma) with gentle shaking. The digested myometrium and endometrium were gently triturated in HBSS with 0.1% BSA (Sigma). The cell suspension was filtered through a double 53-mm nylon mesh and centrifuged at 300 x g for 10 min. Myometrial and endometrial cells were then resuspended in DMEM medium containing 10% FBS and 1% penicillin-streptomycin (Life Technologies) and plated at a density of 1 x 106/ml in eight-well chamber slides. The cells were cultured for 24 h at 37 C in a humidified atmosphere of 95% air-5% CO2 to allow attachment of the cells to the slides. The culture medium was then aspirated, and the cells were washed twice with PBS. The slides were stored at -80 C until they were processed for immunocytochemistry.

Western blot

To prepare solubilized cell membrane extracts, uterine myometrium and endometrium were ground into small pieces and homogenized with a Polytron (Brinkmann Instruments, Westbury, NY) for 15 sec (twice) at speed setting 6 on ice in TRF buffer (50 mm Tris (pH 7.4), 10 mm EDTA,
and 1 mM diethylthiocarbamic acid (DEDTC; Sigma) containing 2 mM octyl glucoside (Sigma) and centrifuged at 30,000 x g for 30 min at 4 C (13). The crude pellets containing cell membranes were sonicated (8 sec cycle; three cycles; Branson Sonifier, Danbury, CT) in 500 µl TON sonication buffer (20 mM Tris (pH 7.4), 50 mM EDTA, and 0.1 mM DEDTC containing 45 mM octyl glucoside). The sonicates were centrifuged at 13,000 x g for 25 min at 4 C. The recovered supernatant was stored at -80 C until electrophoretic analysis. The protein concentration was determined by the method of Bradford (Bio-Rad Laboratories, Richmond, CA).

The solubilized proteins (50-100 µg/ml) were then separated on 10% SDS-PAGE and electrophoretically transferred to nylon membrane (Immobilon) using a Bio-Rad transfer blot cell. The filters for immunostaining were blocked with 2% BSA in 10 mM Tris-Cl buffer containing 0.1% Tween-20. After blocking, the blots were washed three times with wash buffer (5 min each containing 10 mM Tris-Cl and 0.1% Tween-20) and incubated with the polyclonal rabbit anti-OTR antiserum generated by a synthetic dodecapeptide (WQNLRLLKATAAA) corresponding to the third intracellular loop of the rat OTR sequence, which tends to be the area of least homology between receptors of the AVP/OT family (7) (no. 3579; 1:1000 dilution), or with normal rabbit serum or preabsorbed OTR antiserum at room temperature for 1 h. After each antibody incubation, the blots were washed twice in PBS for 10 min each time. Unless otherwise specified, all slides were sequentially incubated at room temperature with each of the following reagents for the time indicated: 1) 3% (vol/vol) H2O2 in PBS for 30 min; 2) 10% (vol/vol) normal goat serum with 5% (wt/vol) BSA in 0.25 M Tris-Cl with 0.15 M NaCl (TBS) for 1 h; 3) polyclonal rabbit anti-OTR antiserum (no. 3579; 1:1000 dilution) at room temperature for 1 h; 4) biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 1 h; 5) avidin-biotin complex (Vector) for 1 h; and 6) 3,3-diaminobenzidine tetrahydrochloride (Sigma), 4 mg/10 ml 0.05 M Tris buffer (pH 7.6), and 10 ml 3% H2O2 for 20 min. After each incubation, the slides were washed with TBS for 15 min, except in step 2. The slides were then counterstained with hematoxylin and mounted.

The specificity of the anti-OTR antibody was controlled by 1) omission of the primary antibody, 2) incubation of the slides or membranes with normal rabbit serum instead of the primary antibody, and 3) incubation of the slides or membranes with the primary antibody preabsorbed with the same synthetic dodecapeptide (50 µg/ml) specifically produced to raise the OTR antibody.

**Results**

**Western blot**

As shown in Figs. 1 and 2, after Western blot analysis, anti-OTR antiserum stained a protein with an approximate molecular mass of 66 kDa in both the myometrium and endometrium. This staining was abolished by incubation with the preabsorbed OTR antiserum or by replacing the OTR antiserum with normal rabbit serum (Fig. 1). Only one major protein species reacted with this OTR antiserum in either myometrium or endometrium (Figs. 1 and 2). OTR protein increased in the myometrial and endometrial samples collected during glucocorticoid-induced labor (P < 0.05; Figs. 2 and 3).

**Northern blot analysis**

Northern blot analysis was used to validate the ovine OTR cDNA probe before using it for the in situ hybridization studies and to measure the changes in OTR mRNA level before and during glucocorticoid-induced labor. Hybridization occurred between the 32P-labeled OTR cDNA probe and the OTR mRNA from ovine myometrium (Fig. 4). There was only a single RNA transcript at 6.6 kilobases, which hybridized with the OTR cDNA probe. OTR mRNA increased significantly (P < 0.001) during glucocorticoid (dexamethasone or betamethasone)-induced labor (Figs. 3 and 4).
OTR IN PREGNANT OVINE UTERUS

Discussion

OT is the strongest uteroton agent known. Although OT is used extensively to induce labor and control postpartum hemorrhage, the precise role of endogenous OT in the initiation of parturition remains unclear. This dilemma in part arises from the lack of consensus about whether the rise in OT in the maternal circulation precedes the actual onset of labor (14). This dilemma is compounded by the arbitrary allocation of a timing for the onset of labor. Currently, a range of characteristics is selected differently by different investigators from among a multifactorial group of interconnected events. These events themselves are regulated by an equally large group of changes that occur in a closely knit sequence as pregnancy progresses. For example, in human term labor as well as in many experimental animal paradigms it has not always been possible to precisely relate fluctuations in the maternal plasma OT concentration to one of the major critical changes that indicates labor, namely the change in patterns of uterine activity that occurs in late pregnancy. In three species of nonhuman primates, for example, it has been shown that myometrial activity switches from low grade contracture activity to contraction activity and back again for several nights before delivery occurs (15-18). Because of the difficulty of following these subtle changes both clinically and experimentally, it has proved difficult to determine whether the increase in circulating concentrations of OT is a physiological initiator of labor. However, a role for OT in the process of labor does not necessarily require an increase in circulating plasma OT concentrations. It is also possible that OT produced locally by endometrium (19) or chorio-decidua (20) exerts its action by means of a pathway different from the classical hormonal pathway. Several studies have demonstrated that myometrial sensitivity to OT is enhanced dramatically during parturition. This increased sensitivity to OT is believed to result from an increase in the concentration of OT (5, 21, 22). Previous studies have extensively characterized the OTR in uterine membrane homogenates by receptor binding assays; however, the histological distribution of OTR in late gestation has not been examined. In addition, the physical properties of the OTR have not been described. Understanding the cellular and tissue distribution of the OTR provides valuable information regarding the underlying mechanism of both the regulation of OTR and its action in the pregnant sheep uterus.

We have simultaneously examined OTR mRNA and OTR protein in the pregnant sheep myometrium and related these changes to the onset of labor induced with two different glucocorticoids. The elevated OTR mRNA concentrations in...
FIG. 5. In situ hybridization and immunolocalization of OTR in the pregnant sheep myometrium and endometrium. Specific silver grains formed by hybridization of OTR cDNA probe with OTR mRNA are observed in myometrial cells (A) and glandular cells in endometrium (B). Positive immunostaining for OTR is present in the pregnant sheep myometrium (C) and the epithelial cells of the gland in endometrium (D). The cultured myometrial (E) and endometrial (F) cells also showed positive staining for OTR. OTR was also present in the smooth muscle cells of myometrial blood vessel (G). Magnification, ×500.

pregnant sheep myometrium associated with labor confirms our previous observations (5). In the present study we have shown that there is a concurrent rise in OTR protein at the same time as this increase in OTR mRNA. These results are consistent with our previous hypothesis that the increased response of sheep myometrium to OT during labor, indicated by the elevated levels of OTR, will promote the effect of OT on the myometrium and contribute to the action by which OT exerts its influence during the process of parturition. The mechanisms that up-regulate OTR production during labor are not very well defined. Several studies have demonstrated that estrogens (1, 22, 23) and glucocorticoids (24) have positive effects in up-regulating myometrial OTR, whereas progesterone (23, 25, 26) and OT (27, 28) can down-regulate OTR. It is, therefore, likely that the sharply increased maternal estrogen concentration (29) and the fall in progesterone that occurs immediately before parturition are together responsible for the induction of OTR expression during labor in the pregnant sheep.

In the present study, we used a new OTR antibody to immunolocalize the OTR in the pregnant sheep myometrium and endometrium. We have validated this OTR antiserum by Western blot, showing that the antiserum specifically hybridized to a protein and that the hybridization was abolished by preabsorbed OTR antibody. Previous studies based on photoaffinity cross-linking and electrophoretic analysis of the purified OTR from two-step chromatography have shown that the functional size of OTR in rabbit myometrium and amnion is 65 kDa (30). An additional band with a molecular mass of 50 kDa was also present in the rabbit myo-
The apparent molecular size of the OTR we observed in pregnant sheep myometrium and endometrium was about 66 kDa, in close agreement with the size estimates of the rabbit and rat mammary gland OTR (31). The molecular size of ovine OTR reported here is bigger than that in human OTR (42.7 kDa; 388-amino acid polypeptide) deduced from the cloned human OTR gene sequence (32). However, the mRNA of ovine OTR determined by Dr. Flint (12) and by us (5) is 2.3 kilobases longer than the OTR mRNA from human uterus. This increased size of ovine OTR compared with human OTR partly results from the additional sequence inserted in the 5'-end of the OTR mRNA. The complete structure of the ovine OTR gene sequence has not been analyzed. Different splicing mechanisms for immature OTR mRNA or different structures of the OTR gene may exist in different species. Such differences would explain the existence of OTR proteins of different sizes observed in different species.

Determination of the precise distribution of OTR in pregnant sheep myometrium and endometrium is essential for a complete understanding of the action of OT in its different target tissues. Specific immunostaining for OTR was mainly associated with myometrial cells in pregnant sheep myometrium, whereas in endometrium, the glands comprised the main site for OTR immunostaining. These results provide histological evidence supporting a biological function of OT in the sheep uterus. OT appears to stimulate myometrial contraction by two parallel mechanisms. The first mechanism involves direct activation of OTR on myometrial cells, with a resultant change in intracellular calcium concentrations and increased myometrial contractility (33). The second mechanism involves indirect stimulation of contraction through the release of stimulatory PGs from the endometrium (34–37). The demonstration of OTR at both of these sites of action and the increased OTR in endometrium during labor further supports these mechanisms, as it is well documented that the interaction of OT with its receptor is essential for the transduction of OT’s effects on its target cells. It was also of considerable interest to observe intense OTR immunostaining in the smooth muscle of medium-sized blood vessels. We hypothesize the OT might act on these receptors to prevent postpartum hemorrhage.

Using in situ hybridization, OTR mRNA was identified in myometrial and glandular cells in the pregnant sheep uterus, confirming the distribution of OTR protein found in immunocytochemical studies. The present study is the first to localize OTR mRNA in the pregnant sheep uterus by in situ hybridization. In situ hybridization of OTR mRNA signal in sheep endometrium was localized mainly in the glandular epithelial cells, consistent with the earlier report that OTR mRNA was expressed in glandular epithelial cells, but not observed in stromal cells (6). However, in contrast to our immunocytochemical and in situ hybridization observations, autoradiographic binding assays have shown that oxytocin-binding sites were also present in the stromal cells (38–41). This difference in OTR distribution may be due to the presence in the stromal cells and glandular cells of binding sites for OT that have a modified biochemical structure and demonstrate binding by autoradiography but do not react with our antibody.

In conclusion, these results identify, for the first time, the precise localization of OTR and its mRNA within the pregnant sheep uterus and provide histological evidence for the physiological roles of OT in regulating myometrial contractility and endometrial PG production in the pregnant sheep uterus. The observed increased expression of OTR and its mRNA in myometrium and endometrium during labor further indicated that OT and its receptor are directly involved in the process of parturition in pregnant sheep.

References

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