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Estradiol and Progesterone Regulate Oxytocin Receptor Binding and Expression in Human Breast Cancer Cell Lines

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The effects of estradiol (E_2) and progesterone on the oxytocin receptor (OTR) were investigated in MCF-7 and Hs 578T human breast cancer cell lines. OTR messenger RNA and protein were identified by reverse transcriptase polymerase chain reaction (PCR) and solution-phase hybridization-RNase protection assay, and Western blot analysis, respectively, in cell lines and in cancerous breast tissue removed from women at mastectomy. Cells were exposed to E_2, progesterone, or vehicle (each steroid, 10^{-10}–10^{-6} *M*) for 24 h and harvested for extraction of RNA. The OTR PCR product was increased by E_2 (10^{-7} *M*, $p < 0.05$, or 10^{-6} *M*, $p < 0.01$ vs control) and decreased by progesterone (control vs 10^{-7} or 10^{-6} *M*, each $p < 0.005$). Hs578T cells were cultured in the presence or absence of E_2 (10^{-6} *M*) or progesterone (10^{-6} *M*) for 24 h and binding was measured. For the E_2-exposed cells, the K_d ($p < 0.05$), and B_{max} ($p < 0.01$) were higher whereas for the progesterone-treated cells the K_d ($p < 0.05$) and B_{max} were lower than control cells. E_2 and progesterone not only regulate OTR expression and binding in normal mammary myoepithelium but also in malignant mammary cell lines.

Key Words: Estrogen; human breast cancer; mammary gland; oxytocin; oxytocin receptor; and progesterone.

Introduction

Oxytocin (OT) that is released into the peripheral circulation is synthesized within the hypothalamus of mammals and stored in the posterior lobe of the pituitary gland *(1)*. One of the biologic effects of OT is milk ejection *(2)*. OT exerts this effect via its receptor (oxytocin receptor [OTR]), which localizes to *myoepithelial* cells in *normal* breast tissue *(3,4)*. The OTR in mammary gland membranes has been shown to correspond to OTR in the uterus *(5,6)*. Estradiol (E_2) and progesterone, respectively, have well-known "upregulatory" and "downregulatory" effects on the expression and binding of *myoepithelial* and uterine OTRs *(3, 4,6–9)*. OTR has also been identified in human mammary cancers, which are typically of epithelial, not myoepithelial, origin. Both human breast cancer tissues removed at mastectomy and human breast cancer cell lines express OTR *(10–16)*. In the present study, we measured OTR binding and the OTR reverse transcriptase polymerase chain reaction (RT-PCR) product in the human breast cancer cell lines MCF-7 and Hs 578T, which were cultured in an E_2- or progesterone-enriched environment

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Results

An amplified band of 397 bp of the PCR product corresponding to bp 1215–1620 of the human OTR gene was identified in normal (from reduction mammoplasty) and cancerous (ductal and lobular cancers) human mammary tissue, in human breast cancer cell lines (both estrogen receptor [ER]–negative Hs 578T, and ER-positive MCF-7 and BT-20), and in normal uterine tissue (from term pregnancy) (Fig. 1), but not in 293 renal cells transformed with adenovirus and K 562 chronic myelogenous leukemia cells (not shown). Solution-phase hybridization ribonuclease protection assay identified a 391-bp mRNA fragment in breast cancer cell lines (Hs 578T, MCF-7, and BT-20) and term uterine tissue (Fig. 2). An antihuman monoclonal antibody, 2F8, to the OTR detected a 70-kDa band by Western blot analysis in human breast cancer tissue removed from a woman at mastectomy, term pregnant rat uterus, MCF-7 and Hs 578T cells that are known to express OTR, but not a human metastatic malignant melanoma cell line, JMK 591.8, which is not known to express the OTR (supplied by Dr. John Kirkwood, University of Pittsburgh) (Fig. 3).

MCF-7 or Hs 578T human breast cancer cell lines were exposed to vehicle or E_2 (10^{-10}–10^{-6} *M*) or progesterone (10^{-10}–10^{-6} *M*) for 24 h and harvested for extraction of RNA. The concentration of E_2 in the medium, prior to addition of the E_2, was nondetectable. The OTR PCR product was increased by E_2 in MCF-7 cells (10^{-7} *M*, $p < 0.05$, or 10^{-6} *M*, $p < 0.01$, vs control) (Fig. 4) and Hs 578T cells (10^{-7} or 10^{-6} *M*, each $p < 0.05$, not shown). E_2 (10^{-7} and 10^{-6} *M*) also increased the expression of bcl-2 in these cells (not shown). Progesterone decreased the OTR PCR product in Hs 578T cells (Fig. 5) (control vs 10^{-7} or 10^{-6} *M*, each $p < 0.005$, Fisher protected least significant difference [PLSD]).

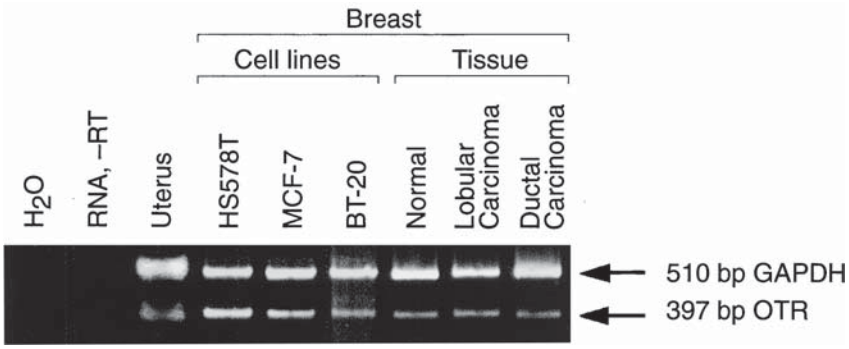

Fig. 1. RT-PCR of OTR. Samples of RNA (1 μg) from human breast cancer cell lines (Hs 578T, MCF-7, and BT-20) and normal and cancerous human breast tissues were reverse transcribed with OTR and GAPDH-specific primers, and each transcript was amplified by PCR. A 397-bp OTR PCR product was detected in the breast cancer cell lines and the normal and cancerous mammary tissues. Lane 1, water; lane 2, RNA without reverse transcriptase; lane 3, human uterine RNA.

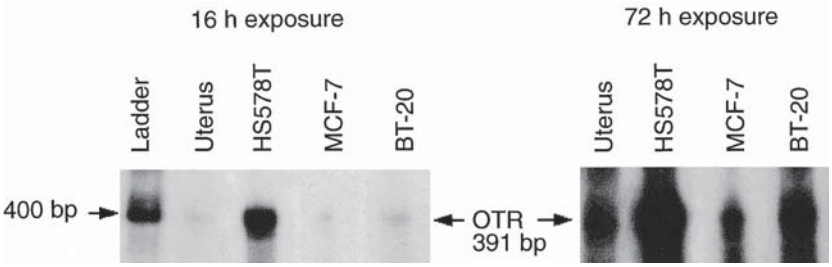

Fig. 2. Solution hybridization/RNase protection assay of OTR. Each lane represents RNA (20 μg) from human breast cancer cells and term uterine tissue. In each sample, an appropriate protected band of 391 bp was identified. Two autoradiograms with exposure times of 16 h and 72 h are shown for the same blot.

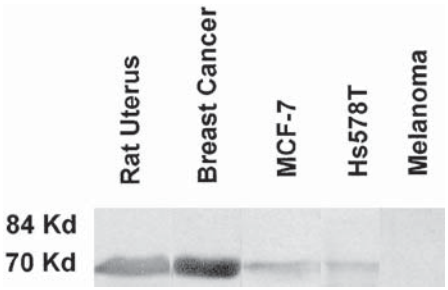

Fig. 3. Western blot analysis of OTR proteins (40–100 μg) from indicated cells and tissues were fractionated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Detection of OTR was carried out with an antihuman monoclonal antiserum (2F8) using an electrochemiluminescence analysis system. A 70-kDa band was detected in pregnant rat uterus, human breast cancer tissue, and MCF-7 and Hs 578T breast cancer cell lines, but not in melanoma cells.

Hs 578T cells were cultured in the presence or absence of E_2 (10^{-6} *M*) or progesterone (10^{-6} *M*) for 24 h and binding was measured. Cells exposed to E_2 had a higher K_d (1.133 ± 0.122 n*M*) than cells not exposed to E_2 (0.755 ± 0.059 n*M*; $p < 0.05$) and a higher B_{max} (459.8 in E_2-treated vs 331.5 fmol/mg of protein in non-E_2-treated cells; $p < 0.001$) (Fig. 6). Cells exposed to progesterone had a lower K_d (0.47 ± 0.05 n*M*) than control cells (0.74 ± 0.04 n*M*; $p < 0.05$) (Fig. 6). The B_{max} was also lower in progesterone-treated (1032.10 fmol/mg of protein) than untreated cells (1491.7 fmol/mg of protein), but this did not achieve statistical significance.

Discussion

We examined the effects of E_2 and progesterone on two different human breast cancer cell lines known to contain the OTR. We found that E_2 increased OTR binding and expression, whereas progesterone had the opposite effect in these cells. Our laboratory is not the first to study the regulation of the OTR in human breast cancer cells. Dexamethasone upregulated the OTR in Hs 578T cells *(16)* and cyclic adenosine monophosphate and phorbol esters induced the OTR in MCF-7 cells *(15)*.

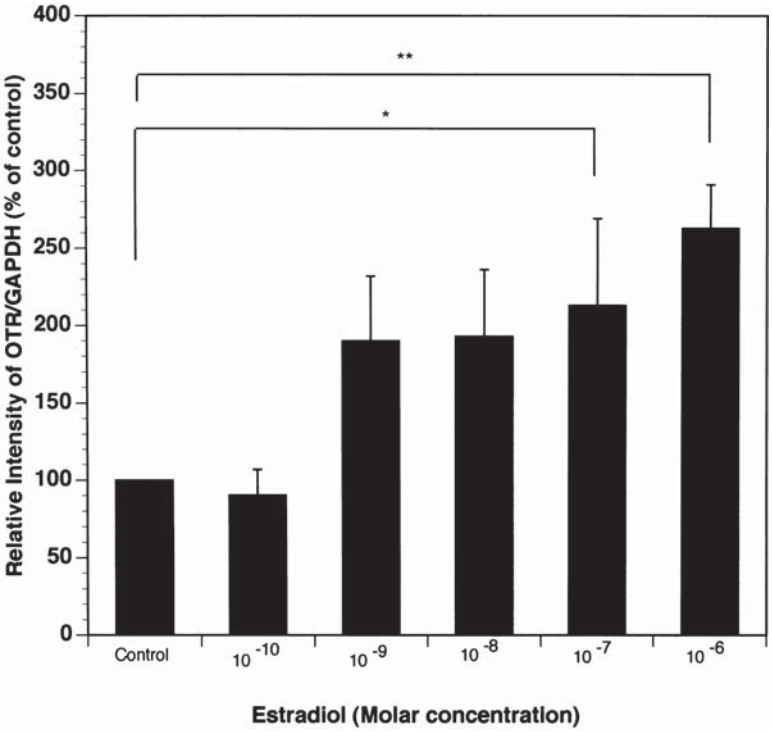

Fig. 4. Expression of OTR RT-PCR product in E_2-treated MCF-7 cells. The OTR PCR product was increased in MCF-7 cells exposed to E_2 (10^{-10}–10^{-6} *M*) for 24 h. (*control vs 10^{-7} *M* E_2, $p < 0.05$; **control vs 10^{-6} *M* E_2, $p < 0.01$, Fisher PLSD). The graph represents the composite of three separate experiments in which triplicate samples were used for each concentration in each of the individual experiments.

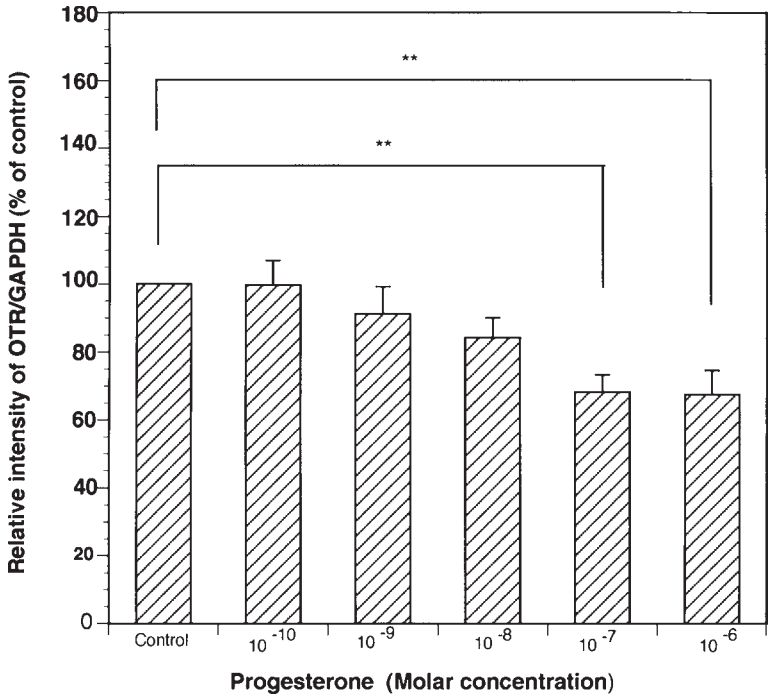

Fig. 5. Expression of OTR PCR product in progesterone-treated HS-578T cells. The OTR PCR product was decreased by exposure of Hs 578T cells to progesterone (10^{-7} or 10^{-6} M) for 24 h (**control vs 10^{-7} or 10^{-6} *M*, each $p < 0.005$, Fisher PLSD). The graph represents the composite of three separate experiments in which triplicate samples were used for each concentration in each of the individual experiments.

We observed significant changes in K_d with both E_2 and progesterone. Although the stimulatory effect of E_2 and the inhibitory effect of progesterone on OTR binding or expression has been well studied in whole-animal models *(3,7–9,17–23)*, few studies have been preformed on cultured cells. Soloff et al. *(18)* observed a fivefold increase in OTR in rat

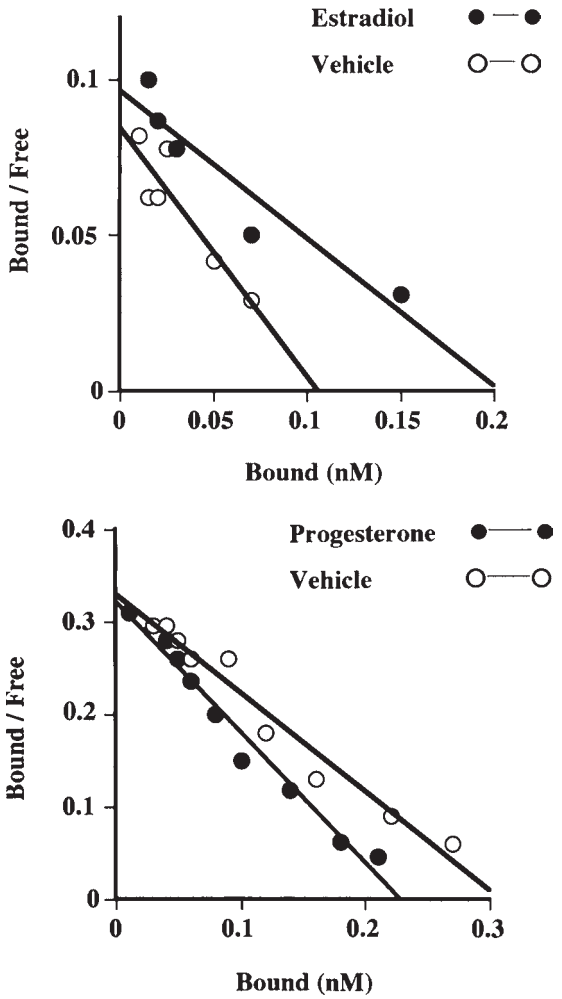

Fig. 6. Effect of E_2 or progesterone treatment on ^{125}I-labeled ornithine vasotocin in HS578T cells. Cells exposed to E_2 (10^{-6} *M*) for 24 h had a higher K_d and B_{max} whereas cells treated with progesterone (10^{-6} *M*) for 24 h had a lower K_d and B_{max} than cells exposed to vehicle. The graphs illustrate a representative experiment. The Scatchard plot measures total binding using IGOR (statfit 1.1) by Wavemetrics.

uterine explants after the addition of as much as 370 n*M* E_2. Progesterone alone had no effect on OTR but in combination reduced OTR by 60%. The effect of E_2 on cultured cells has been less significant. Adachi and Oku *(24)* demonstrated a 40 and 80% increase in OTR in human myometrial cells following incubation with 10^{-8} and 10^{-7} *M* E_2, respectively. This increase was blocked by 50% with the coadministration of progesterone (2.8 m*M*). We observed a similar (43%) increase in OTR in Hs 578T cells with 10^{-6} *M* E_2. Okuda et al. *(25)*, however, were unable to induce an increase in OTR by similar concentrations of E_2 in cultured bovine leuteal cells, which are known to express the OTR. Other paracrine pathways involving different cell types may account for the less-significant increase in OTR with E_2 in cultured cells compared with explant or whole-animal experiments.

Receptor-binding assays and RT-PCR have been the most frequently used methods to study the localization and regulation of the OTR in normal tissues. The OTR has been identified in the target tissues in which OT exerts its effects, such as myoepithelial cells of normal mammary tissues *(3,4)*; myometrial and decidual cells of the normal uterus *(26)*; as well as the pituitary *(27,28)*, brain *(29)*, kidney *(30, 31)*, thymus *(32)*, ovary *(33)*, and testis *(34)*. Because of the well-recognized contractile actions of OT on the myometrium at parturition and the myoepithelial cells during lactation, the regulation of the OTR has been most extensively studied in myometrial and myoepithelial cells. The concentration of uterine myometrial OTRs is increased at the end of pregnancy and rapidly declines following delivery, whereas the concentration of mammary myoepithelial OTRs is increased at term pregnancy but remains high throughout lactation *(3)*. The pregnancy- and lactation-associated changes in OTR are believed to be regulated by the estrogen/progesterone milieu.

A variety of genes that regulate the growth and differentiation of mammary cells are E_2 or progesterone sensitive. At present, it is premature to speculate about the role of OT or its receptor in breast cancer. Ito et al. *(11)* and Sapino et al. *(14)* surveyed human breast cancers removed from women at mastectomy and identified the presence of the OTR in 90 and 70%, respectively, of the samples surveyed. Sapino et al. *(14)* have localized OTR to the cellular membrane of mammary malignancy. In *normal breast* tissue, the OTR is located on the *myoepithelial* cells. Because human breast cancers are typically of epithelial, not myoepithelial origin, the expression of the OTR was unexpected. The function of OTR within cancerous mammary cells is not understood. Presumably, the effects of OT within human breast cancer cells are mediated via the OTR. OT has been reported to inhibit the growth of human breast cancer cells *(10,35)* and decrease the rate of tumor formation *(13)*. Because OT has been reported to inhibit growth and promote differentiation in human breast cancer cells, changes in the abundance or binding of the OTR may in turn regulate mammary differentiation or neoplasia. Upregulation by E_2 and downregulation by progesterone of the OTR may represent a way in which OT exerts its effects on mammary growth and differentiation.

Materials and Methods

Cell Culture

Human breast cancer cell lines (Hs 578T, which is ER negative, and MCF-7 and BT-20, which are ER positive) were purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained in T75 flasks in a 5% CO_2 environment at 37ºC in Dulbecco's modified Eagle's medium (Gibco-BRL Life Technologies, Gaithersburg, MD) supplemented with penicillin (100 IU/mL) and streptomycin (100 mg/mL), and 10% fetal bovine serum.

Cells were passed by treatment with trypsin and used between the Fifth and Fifteenth passages. For experiments, cells were seeded in triplicate in six-multiwell plates. Subconfluent monolayers were washed three times with medium and then incubated with medium for 24 h prior to treatment with E_2, progesterone, or vehicle at specified concentrations. Cells were harvested at the end of an experiment for processing of RNA.

Both the ER-positive MCF-7 cell line and the ER-negative Hs 578T cell line were used in studies investigating the effects of E_2 on the OTR RT-PCR product. Hs 578T cells were used in studies investigating the effects of progesterone on the OTR RT-PCR product and in studies investigating the effects of E_2 and progesterone on binding.

Total RNA Extraction

Total RNA was extracted in ultrapure Trizol Reagent (Life Technologies), and isolated RNA pellets were suspended in diethylpyrocarbonate-treated water followed by treatment with DNase I (0.1 U/μg of total RNA).

Primer Design

Two sets of primers, one for OTR and the other for GAPDH, were synthesized by Life Technologies. OTR-specific sequences were amplified by using the sense strand primer 5' CCTTCATCGTGTGCTGGACG 3' nucleotides and antisense strand primer 5' CTAGGAGCAGAGCACT TATG 3' nucleotides. PCR using this set of primer yields a 397-bp fragment, corresponding to bp 1215–1602 of the human OTR gene *(36)*. The primers have been proven to specifically amplify OTR mRNA and not genomic DNA *(36)*. PCR primers used for amplification of GAPDH-specific sequences were as follows: sense strand, 5' GGCTGA GAACGGGAAGCTTG 3' nucleotides; and antisense strand, 5' TCTAGACGGCAGGTCAGGTC 3' nucleotides. PCR using this set of primer yields a 510-bp fragment.

Reverse Transcriptase Polymerase Chain Reaction

Total RNA (1 μg) was reverse transcribed into first-strand cDNA using 0.5 μg of a15-mer oligo-dt primer (Life Technologies) and superscript TM II RNase (100 U/reaction) (Gibco-BRL) in a final volume of 20 μL of reaction buffer. To ascertain the quality of the RNA preparation, a blank reverse transcription containing all the components except the RT was performed in parallel. Half of the RT first-strand cDNA was used for PCR amplification. To minimize tube-to-tube variation in RT-PCR, RT and PCR master mix were made separately. PCR amplification was performed with a GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT). Samples were placed for 1 min at 94ºC for denaturation, 1 min at 58ºC for primer annealing, and 1 min at 72ºC for extension/synthesis followed by incubation for 10 min at 72ºC. The cycle number was determined so that it identified the optimal number of cycles that allowed determination of both mRNA species yet still remained in the log phase of amplification for both OTR and GAPDH. Following 25 cycles of amplification, an aliquot of PCR fragment was analyzed on 2% agarose gel, 0.5X Tris-borate EDTA buffer, stained with SYBR TM Green I nucleic acid gel stain (FMC Bio Products, Rockland, ME). The bands were visualized and quantified using Storm 860 blue fluorescence/chemifluorescence for the phosphor image system (Molecular Dynamics, Sunnyvale, CA). After staining and scanning, the intensity reading of the OTR product was divided by that of GAPDH.

Ribonuclease Protection Assay

[α^{32}P]-CTP (New England Nuclear, Boston, MA)–labeled antisense riboprobe was transcribed with T7 RNA polymerase and purified via phenol-chloroform extraction. Hybridization was carried out with 2.3×10^6 cpm/tube in a 65ºC heat block overnight. To each tube, RNase A buffer (10 m*M* Tris-HCl, pH 7.5; 5.0 m*M* EDTA, 0.2 *M* NaCl, and 0.1 *M* LiCl containing 40 (μg/mL of RNase and 1000 U/mL of DNase-free RNase T) was added. Protected fragments were isolated on 5% acrylamide–8 *M* urea gel. The gel was dried and exposed to X-ray film at –70ºC. The riboprobe for OTR (provided by Dr. Melvyn Soloff, University of Texas, Galveston) is 453 bp and the protected fragment is 391 bp.

Western Blot Analysis

Tissue or cells were washed with ice-cold phosphate-buffered saline (PBS), lysed in RIPA buffer (50 m*M* Tris, 150 m*M* NaCl, 0.5% deoxycholate, 0.1% SDS and 1% NP-40, pH 8.0) containing leupeptin (10 μg/mL) and PMSF (10 μg/mL), and centrifuged. Soluble proteins were denatured in Laemmli sample buffer. The solubilized proteins (40–100 μg) were separated by SDS-PAGE on a 10% Laemmli minigel and electrotransferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) for 1 h at 100 V with 25 m*M* Tris (pH 8.3), 192 m*M* glycine, and 20% methanol as the transfer buffer. Unbound sites were blocked for 1 h in PBST (PBS plus 1% Tween-20). Transferred proteins were probed with the antihuman OTR monoclonal antibody, 2F8 (Rohto, Osaka, Japan), and visualized by using horseradish peroxidase–conjugated sheep antimouse immunoglobulin and enhanced chemiluminescence kit.

Tissues or cells chosen for study were those previously reported to express the OTR (human breast cancer tissue removed from a woman at mastectomy, term pregnant rat uterus, MCF-7 and Hs 578T cells) or cells that are not known to express the OTR (human melanoma cell line).

Competitive Radioligand Binding Studies of OTR

Cells were washed three times with Hank's solution with 20 m*M* HEPES, pH 7.4, and 1 mg/mL of bovine serum albumin (Hank's balanced salt solution [HBSS]; Sigma, St. Louis, MO). Serial concentrations of 250-μL aliquots of cold ornithine vasotocin (0–49.4 n*M*) dissolved in HBSS and 10 μL of ^{125}I-labeled ornithine vasotocin analog (New England Nuclear) were added to each well. The cells were incubated at room temperature for 3 h with very slow shaking. After

3 h, the incubation medium was discarded. The cells were washed three times with wash buffer to block the reaction. Cells were dissolved by the addition of 500 µL of 1 *N* NaOH. The cell suspension was collected and counts were measured in a gamma counter. Bound and free concentrations of the ligand were calculated from the cycles per minute bound and the total cycles per minute added to each well of the cells. Data were analyzed by Scatchard plot using the iterative program IGOR (WaveMetrics, Lake Oswego, OR).

Statistical Analyses

Samples were measured in triplicate at each experimental point and each experiment was replicated three times. Data are expressed as the mean ± SEM of the three replicate experiments. Multivariate analysis of variance (ANOVA) was used to assess statistical comparisons for OTR among steroid- and nonsteroid-treated cultures. When ANOVA indicated significant differences among groups within an experiment ($p < 0.05$), comparisons between pairs of mean values were made by post hoc Fisher PLSD test. An unpaired *t*-test was used to determine differences in OTR binding between steroid- and nonsteroid-treated cultures. A confidence level of $p < 0.05$ was considered significant.

Acknowledgments

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