Oxytocin Induces the Migration of Prostate Cancer Cells: Involvement of the Gi-Coupled Signaling Pathway

Miao Zhong, Maryam L. Boseman, Ana C. Millena, and Shafiq A. Khan

Abstract

Expression of genes that encode oxytocin (OXT) and vasopressin (AVP) and their cognate receptors in normal and diseased prostates are only partially characterized. Reverse transcription and PCR were used to examine the expression of these genes in normal prostate epithelial and stromal cell lines, k-ras–transformed prostate epithelial cell lines, and in four prostate cancer cell lines. Secreted and cell-associated OXT peptide was measured by an enzyme immunoassay. OXT and its receptor (OXTR) were expressed in all eight prostate cell lines. Cell-associated OXT peptide was also found in all prostate epithelial cell lines except in DU145 cells. Neither AVP nor its cognate receptors (V1a receptor and V2 receptor) were expressed in any prostate cell line examined. These data point to the OXTR as the primary target of OXT and AVP, and suggest that OXT might be an autocrine/paracrine regulator in human prostate. We found that OXT induces the migration of PC3 and PC3M, but not DU145 prostate cancer cells. The effect of OXT is distinct from the epidermal growth factor (EGF)–induced migration of prostate cancer cells, in which ERK1/2 and EGF receptor kinase activities were required. When cells were pretreated with pertussis toxin, the effect of OXT, but not EGF, on cell migration was abolished. Pretreatment with the cyclic AMP analogue, 8-Br-cAMP, did not affect OXT-induced cell migration, which eliminated the nonspecific effect of pertussis toxin. We conclude that a Gi-dependent mechanism is involved in OXTR-mediated migration of prostate cancer cells, and indicates a role for OXTR in prostate cancer metastasis.

Introduction

Oxytocin (OXT) and vasopressin (AVP) are highly homologous peptide hormones that have diverse physiologic functions (1). OXT regulates the contractility of uterine smooth muscle at parturition and mammalian myoepithelium during lactation. AVP, on the other hand, is associated with vasoconstriction, antidiuretic, and adrenocorticotropic hormone–releasing activities. The differences in the physiology of the two hormones result from the activation of a subset of G protein–coupled receptors (GPCR; refs. 2-7). To date, four GPCRs have been identified as targets of OXT and AVP. The AVP type 1a (V1aR), type 1b (V1bR), and OXT receptors (OXTR) activate the GTP-binding protein Gαq to stimulate phospholipase Cβ, whereas the AVP type 2 receptor (V2R) preferentially couples to Gαs to stimulate cyclic AMP (cAMP) accumulation. OXTR was also found to couple to Gi and Gq under specific conditions (8, 9). In turn, these G proteins may couple to distinct signaling pathways in different cell types.

OXT and AVP have been shown to function as autocrine/paracrine regulators of tumor progression in several types of cancers (10-13). OXT acts as a mitogenic factor to induce cell proliferation in Kaposi’s sarcoma, but does not affect tumor cell migration in vitro or angiogenesis in vivo (12). OXT functions as a paracrine factor to induce proliferation and cell migration in human breast tumor–derived endothelial cells (14). On the other hand, it inhibits cell growth in breast cancer cell lines (10). AVP stimulates steroid secretion in adrenocortical tumors that express the endogenous V1aR (13). OXT and AVP are expressed in small cell lung carcinoma and exert mitogenic effects in these cell lines (11). The variable effects of OXT on cell growth are potentially attributed to the distribution of OXTR in different membrane microdomains and are a consequence of coupling to different signaling pathways (15).

OXT treatment has been shown to increase epithelial cell growth, 5α-reductase activity, and contractility in normal prostate (16-18). OXT and AVP have also been implicated in prostate cancer progression (19, 20). Although some clinical studies have shown that high levels of AVP peptide are present in metastatic prostate adenocarcinomas (19), the functional relevance of AVP and the identities of the components for AVP signaling in prostate cancer cells are not known. OXTR has been detected in normal stromal...
and epithelial cell lines, as well as in DU145 prostate cancer cell line (20, 21). It has also been detected in tissues from hyperplastic and neoplastic prostates (20, 21). However, data on the expression of OXT in prostate cells are still controversial (20-22). Recently, atosiban, an OXTR antagonist that blocks the OXT-activated Gq pathway, was shown to inhibit cell proliferation by activating the Gi-mediated pathway in DU145 cells that express the endogenous OXTR (15). Hence, the influence of OXT on prostate cancer progression is dependent on the status of activated intracellular signaling pathways. Moreover, it also seems that ERK phosphorylation is a convergent point of both Gi- and Gq-mediated signal pathways (15). Currently, there is no definitive functional assay available that reflects the coupling of OXTR to the Gi-mediated signaling pathway. In the present study, we show the expression of OXT and OXTR in prostate cells and that OXT stimulates the migration of prostate cancer cells. In addition, we provide evidence which supports the involvement of the Gi-coupled signaling pathway in OXT-induced prostate cancer cell migration.

Materials and Methods

Chemicals

Human OXT, pertussis toxin, PD098059, 8-Br-cAMP, and bovine serum albumin were obtained from Sigma. AG1478 was obtained from Calbiochem, L-371,267 was obtained from Tocris. Rat tail collagen and transwell inserts were obtained from BD Biosciences. Superfine agarose was purchased from U.S. Biological. Cell culture reagents were obtained from BD Biosciences. Moloney murine leukemia virus reverse transcriptase and oligo-dT primer were purchased from U.S. Biological. Cell culture reagents were obtained from BD Biosciences. Superfine agarose was purchased from Tocris. Rat tail collagen and transwell inserts were removed from the plate individually; the cells inside transwell inserts were removed by cotton swabs. The cleaned inserts were fixed in 300 μL of 4% paraformaldehyde (pH 7.5) for 20 minutes at room temperature. Cells on the outside of the transwell insert membrane were stained with 0.2% bovine serum albumin at a cell density of 3 × 10^5 cells/mL. The outside of the transwell insert membrane was coated with 50 μL of rat tail collagen (50 μg/mL) overnight at 4°C. The next day, aliquots of rat tail collagen (50 μL) were added into the transwell inserts to coat the inside of the membranes. The inserts were left to stand for 1.5 hours at room temperature before being washed thoroughly with 3 mL of MEM. Chemoattractant solutions were made by diluting OXT (0.1, 1, 10, and 100 nmol/L) or EGF (3 ng/mL) into MEM supplemented with 0.2% bovine serum albumin at a cell density of 3 × 10^5 cells/mL. The outside of the transwell insert membrane was coated with 50 μL of rat tail collagen (50 μg/mL) overnight at 4°C. The next day, aliquots of rat tail collagen (50 μL) were added into the transwell inserts to coat the inside of the membranes. The inserts were left to stand for 1.5 hours at room temperature before being washed thoroughly with 3 mL of MEM. Chemoattractant solutions were made by diluting OXT (0.1, 1, 10, and 100 nmol/L) or EGF (3 ng/mL) into MEM supplemented with 0.2% bovine serum albumin. MEM containing 0.2% bovine serum albumin served as a control medium. EGF was used as a positive control (27). Control and chemoattractant solutions (400 μL) were added into different wells of a 24-well plate. Aliquots of 100 μL cell suspension were loaded into transwell inserts that were subsequently placed into the 24-well plate. The transwell insert-loaded plate was placed in a cell culture incubator for 5 hours. At the end of the incubation, transwell inserts were removed from the plate individually; the cells inside transwell inserts were removed by cotton swabs. The cleaned inserts were fixed in 300 μL of 4% paraformaldehyde (pH 7.5) for 20 minutes at room temperature. Cells on the outside of the transwell insert membrane were stained using HEMA 3 staining kit (Fisher Scientific, Inc.). The number of stained cells was counted in four nonoverlapping low-power fields of a light microscope, and the average number of cells reflected the cell migration status in each transwell insert. To avoid experimental bias, a systematic random sampling technique was applied in the selection of representative fields, in which sample preparation and handling was executed by different persons.

Results were expressed as migration index, which is defined as the average number of cells per field for test
substance / the average number of cells per field for the medium control. Each experiment was repeated at least three times using a different cell preparation.

**Enzyme immunoassay of OXT peptide**

Six prostate epithelial cell lines (RWPE1, RWPE2, LNCaP, DU145, PC3, and PC3M) were cultured in 100 mm dishes to 80% confluence. Fresh keratinocyte growth medium (without supplements) was added and cells were cultured for 24 hours. The supernatants were collected and centrifuged to remove cellular debris. Attached cells were lysed as described earlier (28). Two independent sets of samples were prepared for the detection of OXT peptide. Concentrations of OXT peptide were measured in the cell lysate and culture supernatant samples using an enzyme immunoassay kit (Assay Designs) according to the instructions provided by the manufacturer. The kit is able to detect OXT peptide levels greater than 11.7 pg/mL. Total protein concentrations were measured in the same assay and intra-assay variation was <10%.

**RNA extraction, reverse transcription-PCR, and quantitative real-time PCR**

Total RNAs were extracted from cells using Trizol and the RNA content was determined with UV spectrophotometer (Bio-Rad). First-strand cDNA was prepared from 2 μg of total RNA using 200 units of Moloney murine leukemia virus reverse transcriptase and 20 μmol/L of deoxynucleotide triphosphate, and 1 unit of Taq in a final volume of 10 μL. PCR samples were denatured at 94°C for 2 minutes, followed by 30 to 35 cycles of the following process: denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 60 seconds. A final extension for 10 minutes was followed up after the cycles were completed.

Nested PCR was also used to amplify OXT cDNA to enhance the specificity and sensitivity. Reaction volumes for both external and internal PCR amplifications were 10 μL. A volume of 0.5 μL of cDNA template and 0.1 μmol/L of external primers were used for external PCR. A volume of 0.4 μL external PCR product was then used as the template for internal PCR along with 0.1 μmol/L of internal primers. PCR products were separated by electrophoresis on a 3% agarose gel containing ethidium bromide. DNA bands were visualized under UV light and images were documented using the Bio-Rad gel imager.

For quantitative real-time PCR, cDNA samples (2 μL) were mixed with 0.2 μmol/L of primers and 2× Sybr Green supermix (Bio-Rad) in a final volume of 25 μL in a 96-well PCR plate and placed in an iCycler thermal cycler (Bio-Rad) for PCR with the following steps: 1, 95°C for 3 minutes; 2, 95°C for 15 seconds; 3, 60°C for 1 minute; 4, repeating steps 2 and 3 for 40 times; 5, 95°C for 1 minute; 6, 55°C for 1 minute; 7, 55°C for 10 seconds; 8, repeating step 7 for 100 times. Melting curves were examined for the quality of PCR amplification of each sample. Calculations were done using the ΔΔCt method (30), in which the expression of OXTR in PrEC was first normalized.

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</table>

The cDNAs were amplified with PCR to detect the expression of OXT, OXTR, AVP, V1aR, V2R, and L19 genes using specific primers listed in Table 1. A cDNA template (2.5 μL) was used for the detection of OXT, OXTR, AVP, V1aR, and V2R, whereas 0.5 μL of cDNA template was used for L19. The cDNA template was mixed with 0.4 μmol/L of primers, 0.5 mmol/L of deoxynucleotide triphosphate, and 1 unit of Taq in a final volume of 10 μL. PCR samples were denatured at 94°C for 2 minutes, followed by 30 to 35 cycles of the following process: denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 60 seconds. A final extension for 10 minutes was followed up after the cycles were completed.
to the expression of L19 and then expressed relative to the corresponding value in other cell lines.

**Statistical analysis**
Data from multiple independent experiments are expressed as mean ± SEM. ANOVA and Duncan’s modified multiple range test were used to examine significance between multiple treatments.

**Results**

**Expression of OXT and AVP and their cognate receptors in prostate cell lines**
To investigate the potential functions of OXT and AVP in prostate cancer progression, steady state mRNA levels of OXT and AVP and their cognate receptors were determined by RT-PCR in several human prostate cell lines. As shown in Fig. 1, OXTR mRNA was present in all cell lines examined (PrSC, PrEC, RWPE1, RWPE2, LNCaP, DU145, PC3, and PC3M). It seemed that OXTR mRNA levels were lower in normal epithelial cell lines (PrEC, RWPE1, and RWPE2) but relatively high in prostate cancer cells, especially in PC3 and PC3M cells. Quantitative real-time PCR was used to determine the relative expression of OXTR in prostate epithelial cell lines. These data confirmed higher expression of OXTR mRNA in advanced prostate cancer cell lines (Fig. 1B).

OXT was detected with PCR from cDNA samples derived from RNA extractions of PrEC, PrSC, and DU145; DNA sequencing verified that the resulting PCR amplicon contained the amplified portion of OXT gene (data not shown). A nested PCR strategy was subsequently applied to enhance the detection specificity and sensitivity. As shown in Fig. 1A, OXT mRNA was present in PrEC, RWPE1, RWPE2, LNCaP, DU145, PC3, and PC3M cells. In contrast, neither AVP nor its cognate receptor mRNAs (V1aR and V2R) were detectable in any of the cell lines used in this study (data not shown). In addition, neither OXT nor OXTR mRNA was detected in the RNA samples processed without reverse transcription (no RT controls) and in the cDNA samples from HeLa cells which have been shown previously to not express OXTR (ref. 29; Fig. 1).

To examine whether OXT gene is translated in these prostate cell lines, the cell lysates and conditioned culture supernatants were collected from RWPE1, RWPE2, LNCaP, DU145, PC3, and PC3M prostate cell lines. The OXT peptide was found in cell lysates from all cell lines except DU145 cells. The levels of OXT peptide (n = 2) in RWPE1, RWPE2, LNCaP, PC3, and PC3M were 47.4 (54.0/40.7), 16.0 (19.0/13.0), 3.1 (3.5/2.7), 13.5 (3.0/24.0), and 129.2 (148.6/109.8) pg/mg protein, respectively. However, the OXT peptide was not detectable in the culture supernatants, presumably due to the sensitivity of the enzyme immunoassay.

**Differential influences of OXT on migration of prostate cancer cells**
OXT was shown to induce the migration of human endothelial cells that originated from different tissues (14, 31). In the present study, we found for the first time that OXT treatment stimulated cell migration in PC3 and PC3M cell lines but not in the DU145 cell line (Fig. 2A). On the other hand, EGF (3 ng/mL) induced cell migration in all three cell lines (Fig. 2A). As shown in Fig. 2A, the basal motility was relatively higher in PC3 and PC3M cells when compared with DU145 cells under the same experimental conditions. EGF treatment significantly increased migration in PC3 cells (2.7 ± 0.16-fold) as well as in PC3M cells (1.8 ± 0.18-fold), OXT also induced a dose-dependent increase in the migration of PC3 and PC3M cells, and the effects of OXT on migration were comparable between these two cell lines (Fig. 2B). A significant increase in cell migration was observed when PC3 and PC3M were treated with OXT at the low dose.
of 1 nmol/L (32). As shown in Fig. 3, the effect of OXT (100 nmol/L) was completely inhibited by the pretreatment of PC3M cells with a specific OXTR antagonist (L-371,267; 1 μmol/L).

Distinct molecular mechanisms underlying the migration of prostate cancer cells induced by activated OXTR versus EGF receptor

Activated ERK1/2 and EGF receptor (EGFR) have been shown to phosphorylate multiple cytoskeleton proteins and to promote cell migration (33, 34). To find out potential involvements of EGFR and ERK1/2 in OXT-induced cell migration, PC3M cells were treated with either an EGFR kinase inhibitor (AG1478; 1 μmol/L) or an MEK inhibitor (PD098059; 20 μmol/L) to prevent ERK1/2 activation. As shown in Fig. 4A, OXT (100 nmol/L) induced an approximately 2-fold increase in the migration of PC3M cells; neither of the inhibitors was able to completely inhibit the OXT-induced migration. EGF (3 ng/mL) induced a 1.9-fold increase in migration of PC3M cells. Pretreatments with AG1478 and PD098059 completely blocked EGF-stimulated cell migration (Fig. 4B).

Gi-coupled pathway mediates the OXTR-induced migration of prostate cancer cells

Because OXTR has been shown to couple to multiple G proteins, including Gq and Gi, the involvement of Gi in OXT-induced cell migration was examined by treating cells overnight with pertussis toxin (100 ng/mL). As shown in Fig. 5, pertussis toxin alone did not affect the basal motility of PC3M cells. However, it completely blocked the effects of OXT on cell migration. Previous studies have reported on the ability of pertussis toxin to inhibit Gq-dependent pathways operating downstream of the activated OXTR (35-37). This inhibitory action is mediated via activated protein kinase A (PKA) rather than being a direct consequence of the inhibition of Gi proteins. To examine the potential influence of PKA activity on OXT-induced cell migration...
migration, PC3M cells were pretreated for 30 minutes with 8-Br-cAMP (0.3 mmol/L), the cell-permeable cAMP analogue that activates PKA. The 8-Br-cAMP pretreatment did not exert any effect on OXT-induced cell migration, which excluded the involvement of PKA in the inhibitory effect of pertussis toxin. Similar effects of pertussis toxin and 8-Br-cAMP were also observed in PC3 cells (data not shown). The possibility of nonspecific effects of pertussis toxin was ruled out by the fact that it did not block EGF-induced cell migration (Fig. 5).

**Discussion**

The expression of genes that encode OXT, AVP, and their cognate receptors in normal and diseased prostates and their biological effects are only partially understood. In the present study, mRNAs of OXT and OXTR were found to be expressed in eight human prostate cell lines. On the other hand, mRNAs of AVP and AVP receptors (V1aR and V2R) were not detected.

The expression of OXT has been extensively investigated in prostate tissues and cells with controversial results (20, 22, 38). Using a nested PCR strategy in the present study, OXT mRNA was found to be present in all prostate cell lines examined (PrSC, PrEC, RWPE1, RWPE2, LNCaP, DU145, PC3, and PC3M). In addition, cell-associated OXT peptide was also detected in several prostate cells. These data, together with previous studies (21, 22), support the expression of OXT mRNA and peptide in human prostate in normal and disease states. AVP and its cognate receptors have been implicated in tumor progression in several types of cancers (11, 13). However, there has been very limited information about their roles in prostate cancer. AVP and AVP receptor mRNAs were not detected by PCR in any of the prostate cell lines examined in the present study. This finding contrasts with observations of some clinical studies, in which high levels of AVP peptide were shown to be present in metastatic prostate adenocarcinomas (19). The absence of AVP receptor expression in the prostate is supported by microarray analysis information from the Gene Expression Omnibus profile database on the National Center for Biotechnology Information web site. These findings exclude their potential importance in prostate functions. Previously, AVP treatment had been reported to increase prostatic muscle tone and induce prostate contraction (17), which might be mediated by the activated OXTR. OXTR mRNAs have previously been detected in specific cell types in prostate tissues of several species (18, 20, 22). The expression of OXTR mRNA in PrSC (present study) supports its reported function to induce prostate gland contraction (17, 21). An increased expression of OXTR was found in prostate neoplastic...
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ent types of cells (41). Hence, it is tempting to speculate that transactivation of EGFR may function as a general mechanism to converge signal inputs from different types of activated G proteins to influence cell migration. Activated OXTR stimulates ERK1/2 phosphorylation by transactivating EGFR through Gβγ liberated from Gq heterotrimeric complexes in different cell lines (35). However, it has recently been shown that transactivation of EGFR is not involved in OXTR-induced migration in human umbilical vein endothelial cells, despite the fact that the OXTR-mediated Gq-phospholipase C pathway is involved (31). In the present study, we observed the differential effects of OXT and EGF on migration of DU145 cells. This data is not consistent with the potential involvement of EGFR in OXTR signaling that leads to cell migration. As additional evidence, OXT stimulated a substantial migration of PC3M cells in the presence of EGFR kinase inhibitor (AG1478) or MEK inhibitor (PD098059) which blocks ERK1/2 activation. Activated ERK1/2 have been shown to interact with and phosphorylate multiple cytoskeletal proteins (33). ERK1/2 activities are also required for EGF-mediated migration in several types of cells (42, 43). In line with these findings, the EGF-induced migration of PC3M cells was effectively blocked by pretreatments with PD098059 and AG1478. Our data suggest that ERK1/2-dependent cell migration does not contribute to the migratory process induced by activated OXTR in prostate cancer cells.

Although activated OXTR relies on a Gq-phospholipase C–dependent pathway to induce migration of human umbilical vein endothelial cells (31), it mediates the migration of PC3 and PC3M cells predominantly through the Gi-dependent pathway because the effect of OXT was abolished by pertussis toxin pretreatment. Pertussis toxin ADP-ribosylates and inhibits Gαi activation. On the other hand, it might influence cell functions through intracellular signaling events other than Gi inhibition (44), such as PKA activation to perturb OXTR signaling through Gq-dependent pathways (35–37). Pretreatment with the cAMP analogue, 8-Br-cAMP, did not affect OXTR-induced migration of PC3 and PC3M cells. This data suggests that PKA activation is not involved in the inhibitory effects of pertussis toxin, and in turn, strongly supports the role of Gi proteins in OXTR-induced migration in prostate cancer cells. In addition, pertussis toxin pretreatment did not inhibit the EGF-induced migration of PC3M cells. This data excludes any nonspecific influence of pertussis toxin on the cell migration process.

In summary, we provide evidence that supports the expression of OXT and OXTR in several types of prostate cancer cells, which is consistent with a potential autocrine/paracrine function of OXT in normal and diseased prostates. The lack of AVP and its receptors suggests that OXTR is the primary target of OXT and AVP in the prostate. Finally, OXT treatment induces the migration of PC3 and PC3M cells, which is mediated by the Gi-coupled pathway. Our data suggest that activation of OXTR might contribute to prostate cancer invasion and metastasis.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We are grateful to Dr. Natalya Klueva and Paulette Dillard for their help in this study.

References

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