Potential regulation of PTH/PTHrP receptor expression in choriocarcinoma cells

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ABSTRACT

Objective: Parathyroid hormone-related peptide (PTHrP) have been found to be expressed in a variety of human tumors. Parathyroid hormone-related peptide is known as the major mediator of humoral hypercalcemia of malignancy, may also regulate placental calcium flux, uterine contraction and fetal tissue development. The purpose of this study is to evaluate the expression of PTH/PTHrP receptor in choriocarcinoma JAR cell line.

Methods: This study was carried out at the Department of Biochemistry, College of Science, King Saud University, Riyadh, Kingdom of Saudi Arabia, between November 2002 and August 2003. Choriocarcinoma JAR cell line treated for 12 and 72 hours with epidermal growth factor, (EGF) (20ng/ml), estradiol, E2 (10^{-8} M), dexamethasone, (DEX) (10^{-8} M) or 1,25 dihydroxycholecalciferol, 1,25 (DHCC) (10^{-8} M). We investigated the expression of parathyroid hormone (PTH)/PTHrP receptor in JAR cell line with these treatments compared with untreated JAR cells. The PTH/PTHrP receptor expression were detected with 3.3nM ^{125}I-PTHrP-34$^{Tyrosine.}$

Results: The expression of the receptors at 12 hours were increased following exposure to EGF, E2 or DEX, whereas 1,25 DHCC inhibited the receptor expression. In further experiments at 72 hours with the same treatments, the receptors expression were remarkably increased with EGF, E2 or DEX, whereas, 1,25 DHCC inhibited the receptor expression in these cells.

Conclusion: These data suggested that in JAR cells, The EGF, E2 and DEX upregulated the PTH/PTHrP receptor expression, whereas the 1,25 DHCC down-regulated the PTH/PTHrP receptor, and the 1,25 DHCC may play an important role as antiproliferative drug for choriocarcinoma.

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Parathyroid hormone-related peptide (PTHrP) was originally identified as the humoral hypercalcemia of malignancy (HHM) causative factor[1-3] and originally isolated from tumor lung cells.[2] The parathyroid hormone (PTH) and PTHrP share many biological actions due to the amino terminus homology of both peptides.[4] The PTHrP gene encodes a mature protein of 141 amino acids, and the alternative splicing of the gene occurs resulting in PTHrP proteins of 139 and 173 residues.[5] The PTH and PTHrP peptides interact with a common G protein-coupled receptor known as PTH/PTHrP receptor.[6-8] Moreover, the PTH/PTHrP receptor is coupled to adenylate cyclase and phospholipase C to generate multiple second messenger including c'AMP and 1,4,5-inositol trisphosphate (IP3)[9] and changes in intracellular calcium.[8] In normal physiology, the common PTH/PTHrP receptor have been identified in myometrium, fetal tissue, placental syncytiotrophoblast brush border and basal plasma membrane in which to modulate potential autocrine, paracrine and endocrine roles in growth and differentiation during pregnancy.[10,11] However,

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different effects of inhibitory analogues of PTH and PTHrP have been observed suggesting that distinct receptors for each peptide may exist in some tissues.[12] Hence, when tumor derived PTHrP enters the circulation, it activates receptors in classic PTH sensitive organs, such as bone and kidney, elicits PTH like activity that gives rise to HHM.[13] Epidermal growth factor (EGF) and steroid hormones is well established to play a vital role in placental development during gestation.[14,15] Despite the understanding of the EGF and steroids hormone roles in pregnancy, no investigations have been performed to examine the regulation effects of these compounds on PTH/PTHrP receptor expression in choriocarcinoma cells. Therefore, the purpose of this work is to investigate the possible effect of EGF, Estradiol (E2), 1,25 dihydroxycholecalciferol (1,25DHCC) or dexamethasone (DEX) in choriocarcinoma cells.

Methods. ***Cell cultures.*** This study was performed between November 2002 and August 2003 at the Biochemistry Department, College of Science, King Saud University, Riyadh, Kingdom of Saudi Arabia. Choriocarcinoma JAR cell line (ECCAC, Salisbury, United Kingdom, (UK)) were grown to 80% confluence in defined media (RPMI) supplemented with 10% fetal calf serum (Imperial Laboratories), 2mM L-glutamine (Sigma), and 1% penicillin, streptomycin and amphotericin (PSA) (Sigma, UK). They were growth arrested for 24 hours before any experiment by replacing with serum free medium. The cells were subcultured into flasks following cell dispersion with 10% trypsin/EDTA solution in calcium free Hanks solution (Sigma, UK) the appropriate phenol red free media with 2.5% charcoal stripped fetal calf serum, and each flask had one of the following treatments, EGF (20ng/ml) (Bachem, UK), E2 (10^{-8}M) (Sigma, UK), 1,25DHCC (10^{-8}M) (Bachem, UK) or DEX (10^{-8}M) (Sigma, UK). Cells were incubated for 12 or 72 hours at 37°C in an atmosphere of 95% air-5% CO_2.

Radioiodination. Human PTHrP-1-34-tyr^{34,} 50μg (Peninsula) was iodinated by the iodogen method using sodium (Na)^{125}I (1mCi) (Amersham, UK).[16] The radiolabeled PTHrP-1-34^{Tyrosine} was separated from free Na^{125}I by G-50 sephadex resin (Pharmacia) chromatography. The specific activity of the iodinated PTHrP-1-34 was calculated as 1.87 x10^{-11}pmole of ^{125}I / 2 x10^{-11} pmole of peptide.

Affinity labeling. Saturation assay was performed for 2 hours with 3.3nM of ^{125}I-PTHrP-1-34^{Tyrosine} with or without additional 200nM unlabeled PTHrP-1-34.[17] JAR cells were treated with EGF (20ng/ml), E2 (10^{-8}), DEX (10^{-8}) or 1,25 DHCC (10^{-8}) then incubated for 12 or 72 hours in an atmosphere of 95% air-5% CO_2. Before photolabeling, the medium was replaced with serum free medium; the cells were incubated for one hour at 37°C in 5% CO_2 in air. Cells were washed twice with ice cold PBS (10mM Na_2HPO_4, 10mM NaH_2PO_4) (pH 7.4) then collected into 1ml PBS (pH 7.4), transferred into an ependorff tube and centrifuged for 5 minutes at 300g, 4°C. The pellets were resuspended with 200ul of PBS (pH 7.4). 3.3nM of ^{125}I-PTHrP-1-34^{Tyrosine} was added into all untreated or treated cells then incubated in ice for 2 hours.

For both saturation and treatment assay, cross-linking was accomplished with N-hydroxysuccinimidyl 4-azidobenzoate HSAB (Pierce, UK) (1mg/500ul in dimethyl sulfoxide) for one hour at 4°C in dark. The reaction was quenched by adding 1ml of ice-cold PBS (pH 7.4) followed by centrifugation at 4000 rpm at 4°C for 10 minutes. The pellets were washed twice with PBS then lysed in fresh ice-cold RIPA buffer (250ul) [50mM Tris (pH 7.5), 150mM NaCl, 100mM NaF, 1mM PMSF, 200uM sodium orthovanadate, 10% glycerol), 1% Triton X-100, 10ug/ml aprotinin, and 0.5% deoxycholic acid]. The cells lysate were standardized according to the protein concentration determined by the Bradford method then sample buffer was added and heated at 80°C for 3 minutes.[18] The samples were loaded as 10ug protein /100ul and separated using 10% SDS-PAGE. The PTH/PTHrP receptor-^{125}I-PTHrP-1-34^{Tyrosine} complex was detected by exposing the gel for one week with RX Fuji medical x-ray film at -70°C. For calibration of molecular weight, the lengths of the migration paths of the single band of the marker proteins and that of the sample in the autoradiography films were measured from the beginning of the resolving gel.

Statistics. All data are representative of 4 independent experiments. Statistical significance was defined using Dunnet's tests. Data is listed as mean ± standard error and the level of significance set at $P<0.01$, and $P<0.001$.

Results. ^{125}I-PTHrP-1-34^{Tyr} cross-linked to one major specifically labeled component of approximately 85 kilo daltons (Kda), which was visualized by autoradiography (**Figure 1**). The formation of the affinity-labeled complex was specifically inhibited by prior exposure with 200nM of unlabeled PTHrP-1-34.

As shown in **Figure 2**, the EGF and DEX caused significant increase in the PTH/PTHrP receptor expression at 12h (47% ± 11, $P<0.01$ and 38% ± 8, $P<0.01$). In contrast, 1,25 DHCC causes a significant decrease (62% ±15, $P<0.001$) in receptor expression compared with untreated cells, whereas E2 had no significant effect on the PTH/PTHrP receptor expression during the first 12 hours treatment. In addition, when the cells were treated

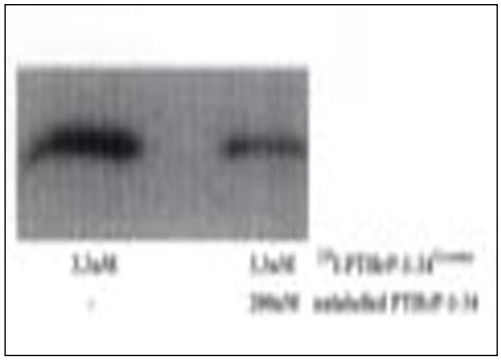

Figure 1 - Saturation assay was performed for 2 hours with 3.3nM of ^{125}I-parathyroid hormone-related peptide-134^{Tyrosine} without or with 200nM unlabeled parathyroid hormone-related peptide-1-34. ^{125}I-parathyroid hormone-related peptide-1-34Tyrosine labeled with parathyroid hormone/parathyroid hormone-related peptide receptor in one major 85kda component in both samples.

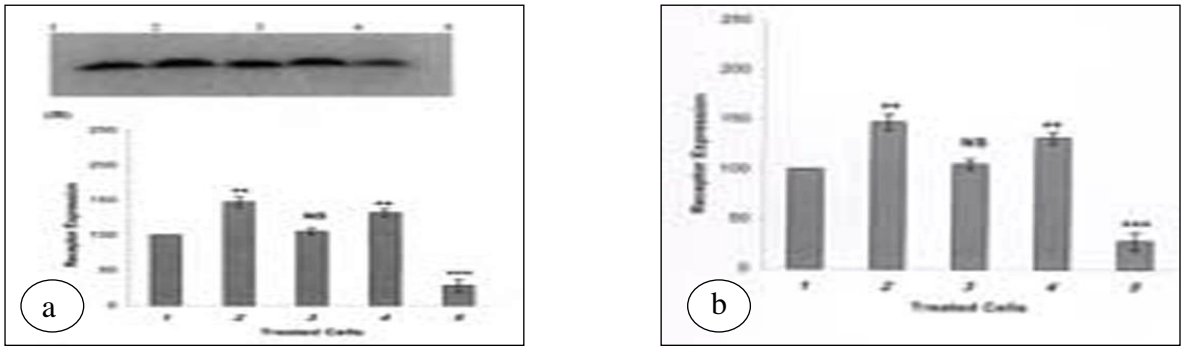

Figure 2 - Effect of epidermal growth factor (2), estradiol (3), dexamethasone (4) or 1,25 dihydroxycholicalciferol (5) on parathyroid hormone/parathyroid hormone-related peptide receptor expression at 12 hours compared with control (1). **(a)** Autoradiography shows a single band in the JAR cells at 85 Kda. **(b)** Scanning densitometry of ^{125}I-parathyroid hormone-related peptide receptor cross-linked to parathyroid hormone/parathyroid hormone-related peptide receptor. The asterisks show statistical significance (***P<0.001, **P<0.01, and NS, not significant, determined by Dunnett's test), compared to the control values.

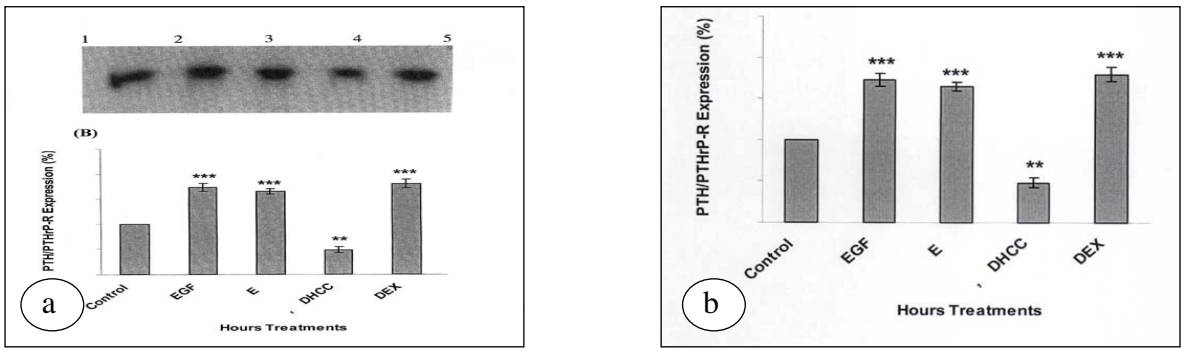

Figure 3 - Effect of epidermal growth factor (EGF) (2), estradiol (E) (3), 1,25 dihydroxycholicalciferol (DHCC) (4) or dexamethasone (DEX) (5) on parathyroid hormone/parathyroid hormone-related peptide receptor expression in JAR cells at 72 hours compared with control (1). **(a)** Autoradiography shows a single band at 85 Kda. **(b)** Scanning densitometry of ^{125}I-parathyroid hormone-related peptide receptor cross-linked to parathyroid hormone/parathyroid hormone-related peptide receptor. The asterisks show statistical significance (***P<0.001) and **P<0.01, determined by Dunnett's test).

with these compounds for 72 hours, EGF, E2 and DEX significantly increase the PTH/PTHrP receptor expression in JAR cells (73% ± 8, $P<0.001$, 65% ±5, $P<0.001$, 80% ± 13, $P<0.001$) compared with untreated cells. The effect of the EGF and DEX appeared to be greater than the effect of E2. In contrast, 1,25 DHCC showed significant decreased (52% ± 6, $P<0.001$) on the PTH/PTHrP receptor expression compared with untreated cells, (**Figure 3**).

Discussion. Parathyroid hormone-related peptide is the major mediator of humoral hypercalcemia of malignancy, may also regulate placental calcium flux, uterine contraction and fetal tissue development.[19] The placental syncytiotrophoblasts is the primary interface between the maternal and fetal circulation. A previous report had been indicated that PTH/PTHrP receptor located on the syncytiotrophoblast at the fetal-facing basal membrane.[10] The presence of PTH/PTHrP receptor in the placenta indicates the potential role of PTHrP in stimulating human placental calcium transport.

The unlabeled PTHrP-1-34 showed its ability to compete with the ^{125}I-PTHrP-1-34^{Tyrosine} on JAR cells. These finding demonstrate the importance of the N-terminal PTHrP for interaction with the PTH/PTHrP receptor. It was well documented that the N-terminal regions of PTHrP is part of the activation domain of the hormone; deletion of amino acids 1-6 diminished greatly or completely abolished agonist-like properties.[17] We examined the effects of EGF, E2, DEX, and 1,25 DHCC on PTH/PTHrP receptor expression in the choriocarcinoma JAR cell line. We indicated that the PTH/PTHrP receptor in JAR cells produced a single band at 85Kda. Similarly, a single receptor band has been reported in HEK-293 cells,[20] COS-7 cells,[21] and other studies found that an analog of PTHrP bound to the PTH/PTHrP receptor in COS-7 cells at 80 Kda.[22] We found that the EGF increase the PTH/PTHrP receptor expression in both treatment periods, and from densitometry analysis, the receptor expression was increased slowly during the first 12 hours treatment compared with the 72 hours treatments. Very little has been known about the interactions between EGF and PTH/PTHrP receptor. The EGF has a number of biological implications in target cells including the differentiation and growth during feto-placental development.[14] The possible effect of EGF on the PTH/PTHrP receptor expression it might be complicated. Estrogens has many biochemical effects through its nuclear receptor, and it has been well established that the estrogen cross-talk and activated second messengers and intracellular signaling cascades such as mitogen activated protein kinase (MAPK) and PI3 which are commonly activated by EGF receptor or coupled to G-proteins receptor.[23]

In addition, our data shown the DEX caused significant increase in the PTH/PTHrP receptor expression at 12 and 72 hours. This result may suggest that the up-regulation of PTH/PTHrP receptor expression was dependent on the interaction of DEX with the glucocorticoid receptor on these cells. Yaghoobian and Drueke[24] have investigated whether DEX changed PTH/PTHrP receptor in ribonucleaic acid (RNA) stability in ROS 17/2.8 cells, and they found that the half life of the receptor mRNA was not changed in response to DEX treatment, but the rate of transcription of the PTH/PTHrP receptor gene was increased two fold in cells treated with DEX. Previous studies in ROS 17/2.8 cells have shown that glucocorticoids enhanced PTH/PTHrP receptor number and steady state mRNA in a dose and time dependent studies,[25,26] and dexamethasone has been reported to upregulate the PTH/PTHrP receptor expression in ROS 17/2.8 cells.[24] These observations therefore suggested that the activation of PTH/PTHrP receptor gene transcription is responsible for increasing mRNA levels resulted in higher levels of express surface receptors. The E2 treated cells have shown that the PTH/PTHrP receptor expression is slow during the first 12 hours, but when the treatment exceeded to 72 hours the E2 over express the PTH/PTHrP receptor expression in JAR cells. In ovariectomized rats, estradiol caused a temporary decline in uterine PTH/PTHrP receptor mRNA levels after 2 and 4 hours treatments, but after 24 hours treatments the level of PTH/PTHrP receptor mRNA was increased.[27] However, in kidneys from ovariectomized rats, the PTH/PTHrP receptor mRNA levels were not affected by estradiol treatment when RT-PCR, in situ hybridization, and cAMP assays were applied.[28]

In this study, 1,25 DHCC caused a significant down-regulation of PTH/PTHrP receptor expression compared with untreated cells. Although our data shows a interested role for 1,25DHCC as inhibitor for PTH/PTHrP receptor expression in JAR cells, no information is known yet about how this compound effect on modulating the PTH/PTHrP receptor gene expression. Consistent with previous studies, 72 hours treatment with 1,25 DHCC shown a marked inhibition of PTH/PTHrP receptor expression in ROS 17/2.8 cells.[29] It has been reported that when normal human keratinocytes treated with 1,25 DHCC for 72 hours, the PTH/PTHrP receptor mRNA levels were undetectable.[30] Moreover, antiproliferative effects of 1,25 DHCC have been documented in many tumors like breast, myeloid leukemia and in prostate cancer.[31,32,33] These findings support the notion that PTH/PTHrP receptor plays a physiological role in the uteroplacental unit and

demonstrate that placental syncytiotrophoblast could be a useful model for studying the regulation of PTH/PTHrP receptor expression. Thus this work clearly demonstrates that the control of PTH/PTHrP receptor in JAR cells is a poorly understood phenomena. However, evidence is accumulating that this ligand/receptor pathway may act as a hormonic modulator of proliferation and metastasis of cancer cells, and this modulator of these mechanisms may be valuable in the treatment of cancer.

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