

Original Article

Differential effects of estrogen and estrogen receptor antagonist, ICI 182 780, on the expression of calbindin-D9k in rat pituitary prolactinoma GH₃ cells

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Abstract: Objective: To detect the effects of 17 β -estradiol (E2) on the expression of calbindin-D9k (CaBP-9k) in pituitary GH₃ cells, and to determine the antagonistic effect of a selective estrogen receptor (ER) antagonist (ICI 182 780) on CaBP-9k expression. Methods: A rat pituitary prolactinoma cell line (GH₃ cells) was used in an *in vitro* model. The localization of CaBP-9k in GH₃ cells was observed by immunofluorescence. GH₃ cells were cultured with the addition of E2 medium for 24 hours. The levels of CaBP-9k mRNA and protein expression in different groups were analyzed by RT-PCR and Western blot analysis. The ER antagonist, ICI 182 780, was added to GH₃ cells before E2 (10⁻⁸ M) at a concentration of 10⁻⁶ M to investigate the regulation of an ER-mediated pathway on CaBP-9k expression. Results: E2 had a stimulatory effect on CaBP-9k expression of GH₃ cells in a dose-dependent manner; the level of CaBP-9k expression was higher when treated with a higher concentration of E2. ICI 182 780 suppressed the stimulatory effect of E2 on CaBP-9k expression in GH₃ cells. The level of CaBP-9k expression was significantly reduced by co-administration of E2 with ICI 182 780 in GH₃ cells. The immunoprecipitation results confirmed that CaBP-9k interacts directly with ER α , and E2 increases the interaction between CaBP-9k and ER α . Conclusion: Estrogen induces CaBP-9k expression via an ER α -mediated pathway and CaBP-9k directly combines with ER α , suggesting that CaBP-9k is involved in the biological effects mediated by an ER pathway in GH₃ cells.

Keywords: GH₃ cells, estrogen, estrogen receptor, ICI 182 780, CaBP-9k

Introduction

Calbindin-D9k (CaBP-9k) is a member of the family of calcium-binding proteins with a high affinity for calcium; CaBP-9k is expressed in the intestines, kidneys, lungs, bones, uterus, placenta, and pituitary gland [1, 2]. CaBP-9k, which is involved in calcium absorption and transfer, is regulated at the transcriptional and post-transcriptional levels by 1,25-dihydroxy vitamin D3 in the intestines [3], and sex steroid hormones in the uterus and placenta [4]. Calcium is an important element in the process of cell signaling and maintenance of cell function, therefore CaBP-9k may be an important element of cellular signal transduction pathways that regulate cell growth, survival, differ-

entiation, and apoptosis, and participate in several important biological processes [5].

The GH₃ cell line, which is a well-established rat pituitary cell line sensitive to estrogen stimulation, is often used as an *in vitro* research model for pituitary prolactinomas. GH₃ cells have been shown to express a high level of estrogen receptor (ER), which plays an important role in GH₃ cell proliferation, apoptosis, and prolactin secretion [6]. Recent studies have shown that the level of CaBP-9k expression in GH₃ cells is regulated by exogenous estrogen *in vitro*, and ER may be involved in the regulatory mechanism [6-8]; however, the role of CaBP-9k in pituitary adenoma tumorigenesis and the underlying regulatory mechanism is unclear. In the present

Estrogen and estrogen receptor antagonist and pituitary prolactinoma

Table 1. Primer sequences and reaction conditions

Primer pair (5'-3', forward and reverse)	T _m (°C)	Cycles	Size (bp)
CaBP-9k			
F: ATG AGC GCT AAG AAA TCT	55	30	287
R: TCA TTC TGATAA CTT TTT GA			
GAPDH			
F: AAC GGA TTT GGC CGT ATT	54	25	300
R: AGC CTT CTC CAT GGT GAA GA			

study we used the GH₃ cell line as an *in vitro* model of pituitary adenomas to determine the effects of estrogens on the induction of CaBP-9k mRNA and protein, and to determine the possible role of ER in the regulation process. GH₃ cells were treated with 17 β -estradiol (E2). The effects of E2 on the induction of CaBP-9k mRNA and protein were examined by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and Western blot assays. Additionally, an ER antagonist (ICI 182 780) was used as an inhibitor of ER to examine the potential involvement of the ER pathway in the induction of CaBP-9k in GH₃ cells. Co-immunoprecipitation was used to determine if CaBP-9k interacts directly with ER and whether or not the expression of CaBP-9k in GH₃ cells is related to an ER pathway.

Materials and methods

Cells and reagents

The rat pituitary cell line, GH₃, was purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). Dimethyl sulfoxide (DMSO) and 17 β -estradiol (E2) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). The ER antagonist, ICI 182 780, was obtained from Tocris (Ellisville, MO, USA). Dulbecco's modified Eagle's medium (DMEM), phenol red-free DMEM, and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, USA). Trizol reagent was purchased from Invitrogen Life Technologies. The cell lysis liquid, RIPA, and a BCA Protein Assay kit were obtained from Thermo (Rockford, IL, USA). Rat monoclonal antibodies against CaBP-9k, ER α , and ER β , anti- β -actin antibody, horseradish peroxidase-conjugated secondary antibody, and FITC-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture and treatment

GH₃ cells were maintained in DMEM medium containing 10% FBS (Gibco) and 100 U/ml of penicillin-streptomycin (Gibco) in a 37°C shaker containing 5% CO₂ and dispersed in a 6-well plate. When the cells grew to 70%~80% fusion, the culture was changed to a phenol red-free DMEM medium containing 5% activated car-

bon-FBS glucan without hormone and 100 U/ml penicillin-streptomycin. The cells were continuously cultivated in this culture for 5 days before use.

Experiment 1: GH₃ cells were treated with different concentrations of E2 (10⁻⁸, 10⁻⁹, and 10⁻¹⁰ M). E2 was dissolved in 0.1% (vol/vol) DMSO, and the vehicle control group (DMSO) was set up. Total RNA or protein was extracted 24 h after administration of E2.

Experiment 2: A group of GH₃ cells was first given ICI 182 780 (10⁻⁶ M) 30 min before E2. The vehicle control group and a separate E2 group (10⁻⁸ M) were set up. Cells were collected after 24 h for total RNA or protein extraction. Each group of experiments was repeated 3 times.

Immunofluorescence assay

GH₃ cells were cultured in 24-well plates, stained with immunofluorescence when the cells reached 50% fusion by adherent growth, washed 3 times with pre-warmed PBS, fixed with 4% formaldehyde for 30 min at room temperature, washed 3 times with PBS, washed 3 times with PBS after a 5-min permeabilization with 0.2% Triton X-100, and sealed with 5% bovine serum albumin (BSA) at room temperature for 30 min. Goat anti-rat CaBP-9k antibody was added, the plates were placed in a wet box, incubated overnight at 4°C, and washed 3 times with PBS. Donkey anti-goat FITC antibody was added, incubated for 30 min at room temperature without light, washed 3 times with PBS, stained with 1 ml DAPI working liquid mu (1 μ g/ml) for 10 min avoiding light, washed 3 times with PBS, and observed under fluorescence microscopy.

RT-PCR

Total RNA of the cells was extracted using the Trizol method. The concentration of RNA was

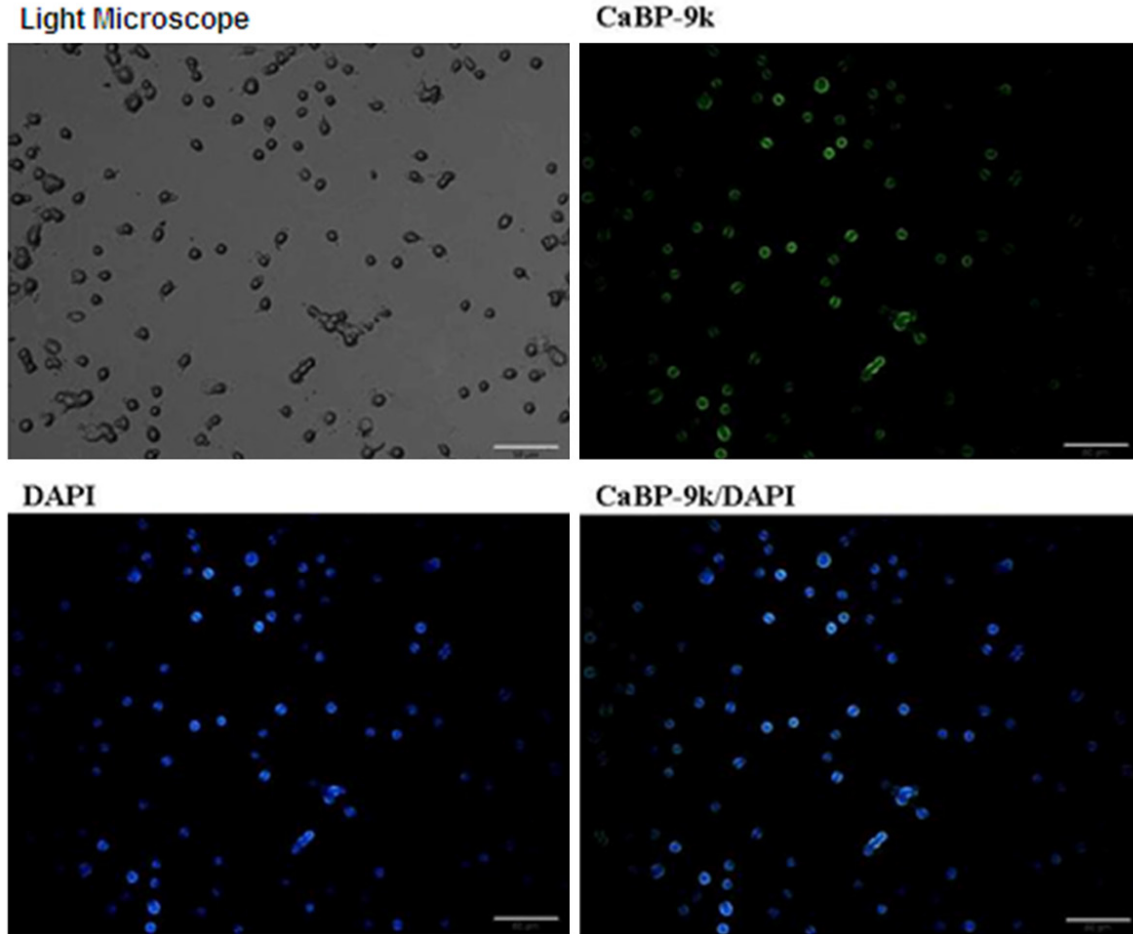


Figure 1. Detection of CaBP-9k expression in GH3 cells by immunofluorescence.

detected using a microplate reader, with purity determined by the ratio of A260/A280. TaKaRa RT-M/MLV kits were used for the reverse transcription reaction. The total reaction volume was 25 μ l (total RNA volume, 1 μ g; 20 μ M oligo [dT; 2 μ l]; and DEPC, 3 μ l). The mixture was heated at 72°C for 5 min after blending, placed on ice immediately for 5 min, and M-MLV (1 μ l), 5X buffer (5 μ l), dNTP (2 μ l), RNase inhibitor (1 μ l), and DEPC (8 μ l) was added, and put into the PCR amplifier for reaction after blending at 42°C for 1 h and 72°C for 15 min. The mixture was placed on ice for cooling for 20 min, and the cDNA templates were obtained. The PCR reaction was performed according to the TaKaRa Ex-Taq DNA polymerase instructions. The reaction conditions were as follows: 95°C denaturation for 5 min; 95°C for 30 s; annealing for 30 s; 72°C extension for 45 s; and 72°C extension for 10 min after reaching the number of cycles. Different primers, the annealing tem-

perature, and the cycle numbers are shown in **Table 1**.

Western blot analysis

The cultured cells were collected after 24 h, counted (1×10^6 cells), the RIPA lysate was added for ice bath cracking for 30 min, centrifuged at 12000 r/min (centrifugal radius = 13.5 cm) and 4°C for 30 min, the supernatant was collected, and the protein concentration was determined by the BCA method. Following SDS-PAGE electrophoresis, the proteins were transferred to PVDF membranes, sealed in 5% BSA at room temperature for 2 h, and thrice-washed with TBS-T for 10 min each. Goat anti-rat CaBP-9k antibody (working concentration = 1:200) was added with β -actin as an internal reference, and incubated overnight at 4°C. Donkey anti-goat IgG-HRP antibody (working concentration = 1:2000) was added, gently

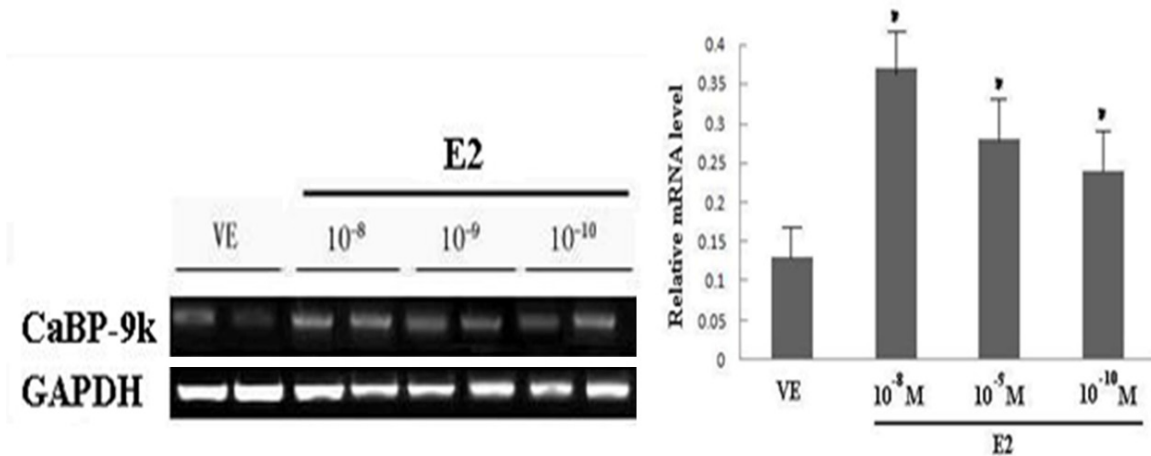


Figure 2. Detection of the effect of E2 on the level of CaBP-9k mRNA expression in GH3 cells based on RT-PCR.

shaken for 2 h at room temperature, and the membrane was washed. An ECL chemiluminescence detection kit was used to detect hybridization signals. Quantity one software was used to detect the gray degree of the strip. The relative ratio of each group was measured according to the gray degree of the internal reference β -actin. Each experiment was repeated three times and the results were averaged.

Co-immunoprecipitation

Cells in the logarithmic growth phase were selected and RIPA lysis buffer and protease inhibitors were added for ice bath cracking for 30 min, centrifuged at 12000 r/min (centrifugal radius = 13.5 cm) for 30 min at 4°C, the supernatants were obtained, and the protein concentrations of a small amount of supernatant were determined using the BCA method. Twenty μ l of Protein G agarose beads (PBS formulated as a 50% concentration) were added to every 400 μ g of cell lysate supernatant, incubated for 60 min at 4°C to remove non-specific hybrid protein, centrifuged at 12000 r/min (centrifugal radius 13.5 cm) for 30 s at 4°C, and the supernatant was collected. Tw μ g of goat anti-rat Calbindin-D9k antibody (IP antibody) was added to 500 μ l of total protein, and the antigen-antibody mixture was gently shaken and incubated overnight at 4°C. The next day, 20 μ l of Protein G was added to capture the antigen-antibody complex, gently shaken at 4°C for 3 h, centrifuged at 12000 r/min (centrifugal radius = 13.5 cm) for 30 s at 4°C, the supernatant was discarded, and thrice-washed with cold RIPA buffer (without a protease inhibi-

tor). The supernatant was completely exhausted, the loading buffer was added, boiled for 5 min at 100°C, and the proteins were separated by SDS-PAGE, transferred to a PVDF membrane, sealed with 5% BSA for 1 h at room temperature, the membrane was thrice-washed with PBS-T, rabbit anti-rat ER α antibody was added, and incubated overnight at 4°C. The membrane was thrice-washed with PBS-T, goat anti-rabbit HRP antibody was added, and incubated at 37°C for 1 h. The membrane was thrice-washed with PBS-T, the ECL reagents were added to the PVDF membrane, and placed in the ECL chemiluminescence analyzer. Images were acquired.

Statistical analysis

All experiments were repeated ≥ 3 times. SPSS 18.0 software was used for statistical analysis. The Student's t-test was used for comparison between the two sets of data, and ANOVA analysis was used for group comparison. A $P < 0.05$ was considered statistically significant.

Results

Detection of CaBP-9k expression in GH₃ cells by immunofluorescence

As shown in **Figure 1**, green phosphor was detected on GH₃ cells, which represented the expression of CaBP-9k. GH₃ cells were simultaneously stained with DAPI in the nuclei. The pictures were superimposed. It was shown that CaBP-9k may be expressed in the cytoplasm.

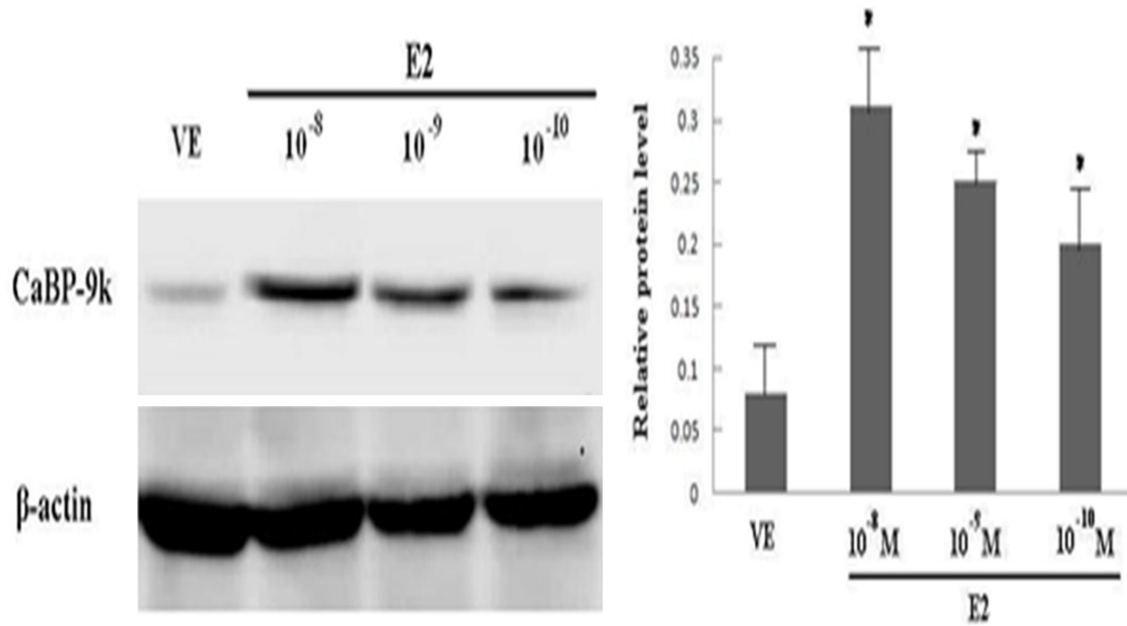


Figure 3. Detection of the effect of E2 on the level of CaBP-9k protein expression in GH3 cells based on Western blot analysis.

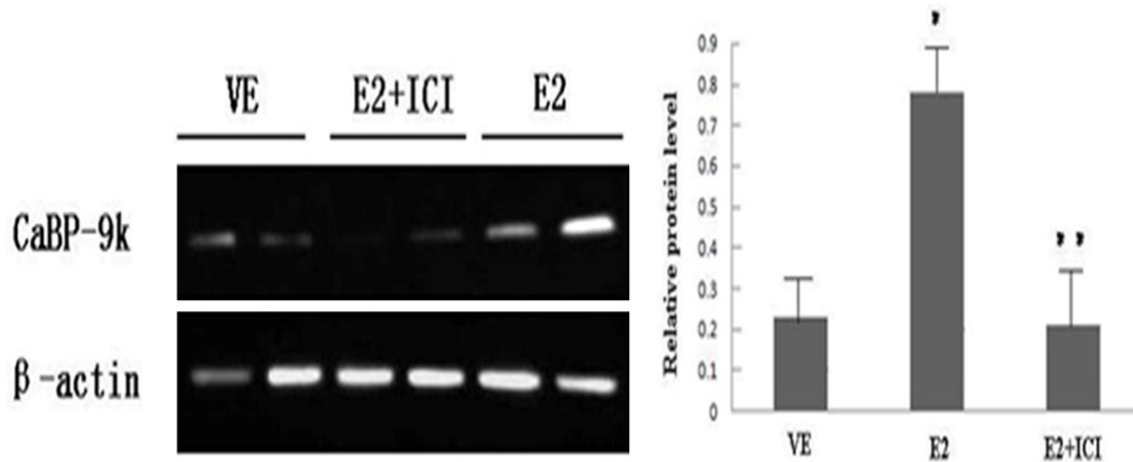


Figure 4. Detection of the antagonistic effect of ICI 182 780 on E2-induced mRNA expression of CaBP-9k in GH3 cells by RT-PCR.

Analysis of the differential effect of E2 on the level of CaBP-9k expression in GH₃ cells based on RT-PCR and Western blot analysis

GH₃ cells were treated with different concentrations of E2 (10⁻⁸, 10⁻⁹, and 10⁻¹⁰ M) for 24 h. The vehicle control group (VE) was set up. Based on RT-PCR and Western blot analysis, the levels of CaBP-9k mRNA and protein expression increased when treated with higher concentrations of E2. **Figure 2** shows the results of RT-PCR. Western blot results are shown in **Figure 3** (*indicates $P < 0.01$).

Investigation of the antagonistic effect of ICI 182 780 on E2-induced CaBP-9k expression in GH₃ cells through RT-PCR and Western blot analysis

VE was the vehicle control group. The E2 group was given 10⁻⁸ M E2. The E2 + ICI group was given 10⁻⁶ M ICI 182 780 30 min before administration of E2; the delivery time was 24 h. The level of CaBP-9k expression in each group of GH₃ cells was detected by RT-PCR and Western blot analysis, as shown in **Figures 4** and **5**. The results showed that ICI 182 780 can inhibit

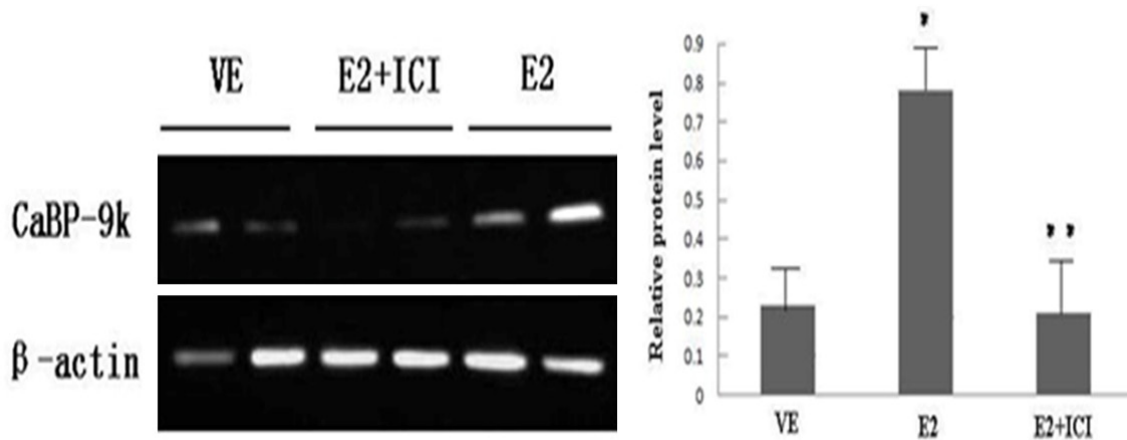


Figure 5. Detection of the antagonistic effect of ICI 182 780 on E2-induced protein expression of CaBP-9k in GH3 cells by Western blot analysis.

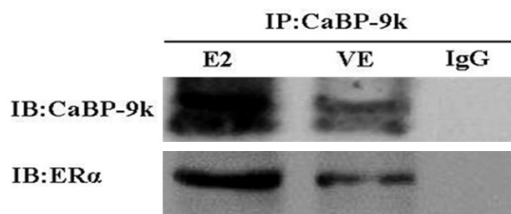


Figure 6. Analysis of interactions between CaBP-9k and ERα by co-immunoprecipitation.

E2-induced CaBP-9k expression at the genetic and protein levels. The E2 group was compared to the VE group (*represents $P < 0.01$; the E2 + ICI group was compared to the E2 group, **means $P < 0.01$).

Analysis of interactions between CaBP-9k and ERα by co-immunoprecipitation

It has been shown that estrogen plays an important role in the occurrence and development of prolactinomas [6], which is closely related to the activation of ER signal transduction pathways. Although numerous studies have revealed that ER signaling pathways play a key role in the occurrence, development, and drug resistance of prolactinomas, the exact mechanism remains unclear. Our previous *in vitro* experiments involving GH₃ cell lines showed that E2 can promote the proliferation of GH₃ cells and PRL release through ER signal transduction pathways (mainly ERα), whereas the ER antagonist, ICI 182 780, can inhibit E2-induced cell proliferation and PRL release [10].

The present study showed that the level of CaBP-9k expression is closely related to E2. As the selective antagonist of ERα, ICI 182 780 can antagonize the effect of E2-induced CaBP-9k expression of GH₃ cells. Therefore, we analyzed the interaction between CaBP-9k and ERα by immunoprecipitation. The protein of interest gained by the immunoprecipitation method was treated with the CaBP-9k antibody and purified through combination with Protein G, denatured, separated by electrophoresis, and transferred to a membrane. Then, the ERα antibody was added to create a hybridoma. The interaction between the two was analyzed. The results are shown in **Figure 6**. In the GH₃ cells, lysis buffer of the E2 and vehicle control groups, protein bands were hybridized in both immune co-precipitation complexes consistent with the target protein, suggesting that CaBP-9k and ERα can combine with each other directly in GH₃ cells, and after the administration of E2, the interaction between the two may increase.

Discussion

Prolactinomas are the most common pituitary adenoma, accounting for 45% of pituitary adenomas and 40%-60% of hormone-secreting tumors. Currently, the pathogenesis of prolactinomas is unclear; however, the present study confirmed that estrogen plays an important role in the occurrence and development of prolactinomas, which may due to the activation of ER pathways [6, 10]. In our previous research, we studied the effect of estrogen and ER on the proliferation of prolactinoma cells, the secre-

tion of prolactin, and the function of ER antagonist in the treatment of prolactinomas. Based on the *in vitro* experiments of the GH₃ cell line, we found that estrogen promoted the proliferation of prolactinoma cells and pituitary prolactin release significantly through ER signal transduction pathways (mainly ER α), while the selective ER antagonist, ICI, 182 780, inhibited estrogen-induced GH₃ cell proliferation and prolactin release. A study abroad also confirmed that estrogen and ER play an important role in the proliferation and apoptosis of a prolactinoma cell line, and prolactin secretion [6].

Based on the current study we found that the level of CaBP-9k mRNA and protein expression in GH₃ cells was increased by estrogen, while antagonized by the ER antagonist, ICI 182 780, suggesting that ER plays a decisive role in the regulation of CaBP-9k expression. We further analyzed the interaction between CaBP-9k and ER α by co-precipitation and showed that CaBP-9k and ER α combines and interacts directly in GH₃ cells, and the interaction between CaBP-9k and ER α may increase via E2. This suggests that the expression of CaBP-9k in pituitary adenoma is related to the effect of estrogen, which is most likely mediated by an ER α pathway.

Why CaBP-9k was highly expressed in prolactin cells with active prolactin secretion and proliferation? What role does CaBP-9k play in cell proliferation, secretion of prolactin, and tumor invasive growth, and how does CaBP-9k function? From a comprehensive analysis of the relationship between estrogen, ER, and CaBP-9k in GH₃ cells, and the fact that the level of CaBP-9k expression in GH₃ cells is increased significantly while inducing GH₃ cell proliferation and prolactin secretion by estrogen *in vitro*, we can determine the relationship between CaBP-9k expression in GH₃ cells and estrogen. Therefore, we suggest that CaBP-9k may be involved in the downstream biological effects brought out by ER-mediated signaling pathways in GH₃ cells, such as cell proliferation, apoptosis, and prolactin secretion.

The regulation of prolactinomas by estrogen depends on the expression of ER, which is mainly expressed in the nucleus, with a small part expressed in the cell membrane. ER in the nucleus and membrane are interrelated. Estrogen can directly enter the nucleus to combine with the nuclear estrogen receptor (nER), act on the specific binding sites of the target

gene-estrogen response element (ERE), and initiate gene transcription. Pathways which act through nER are referred to as classical or genomic pathways, while estrogen in combination with the estrogen receptor membrane (mER) and mER activates the kinase cascade with the G protein-coupled signal directly or indirectly act through mER and are referred to as non-classical or non-genomic pathways.

In prolactin cells, estrogen has been shown to activate prolactin mRNA transcription through ERE, promote prolactin cell proliferation, and regulate the storage and release of prolactin, whereas the ERE is located upstream of the 5'-regulatory region [11, 12]. According to the literature, the CaBP-9k gene contains an incomplete palindromic sequence with 15 base-pairs, which is highly homologous with ERE. This sequence is positioned between exon 1 and intron A nucleotides +51 to +65 in the CaBP-9k gene. The interaction between this region and ER α may be the target where estrogen regulates CaBP-9k mRNA promoter activity and its level of expression, i.e., mRNA expression of CaBP-9k is regulated by ER α -ERE pathway. It suggests that CaBP-9k may regulate cell proliferation and secretion of prolactin cells through the action mode of genomic estrogen. What signal transduction pathway estrogen has taken to affect the occurrence and development of prolactinomas and what role CaBP-9k has played will be the subjects of future studies.

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Disclosure of conflict of interest

None.

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