Expression of Ectodermal Neural Cortex 1 and Its Association with Actin during the Ovulatory Process in the Rat


Ectodermal neural cortex (ENC) 1, a member of the kelch family of genes, is an actin-binding protein and plays a pivotal role in neuronal and adipocyte differentiation. The present study was designed to examine the gonadotropin regulation and action of ENC1 during the ovulatory process in immature rats. The levels of ENC1 mRNA and protein were stimulated by LH/human chorionic gonadotropin (hCG) within 3 h both in vivo and in vitro. In situ hybridization analysis revealed that ENC1 mRNA was localized not only in theca/interstitial cells but also in granulosa cells of preovulatory follicles but not of growing follicles in pregnant mare’s serum gonadotropin/hCG-treated ovaries. LH-induced ENC1 expression was suppressed by a high dose of protein kinase C inhibitor RO 31-8220 (10 μM) but not by low doses of RO 31-8220 (0.1–1.0 μM), suggesting the involvement of atypical protein kinase C. ENC1 was detected in both nucleus and cytoplasm that was increased by LH/hCG treatment. Both biochemical and morphological analysis revealed that LH/hCG treatment increased actin polymerization within 3 h in granulosa cells. Interestingly, ENC1 physically associated with actin and treatment with cytochalasin D, an actin-depolymerizing agent, abolished this association. Confocal microscopy further demonstrated the colocalization of ENC1 with filamentous actin (F-actin). The present study demonstrates that LH/hCG stimulates ENC1 expression and increases F-actin formation in granulosa cells. The present study further shows the physical association of ENC1 and F-actin, implicating the role of ENC1 in cytoskeletal reorganization during the differentiation of granulosa cells. (Endocrinology 150: 3800–3806, 2009)

The LH surge in preovulatory follicles plays an important role in causing ovulation, a release of fertilizable oocyte, and initiating terminal differentiation of granulosa cells into luteal cells (1). Many genes required for luteinization are stimulated by the LH surge, including the transcription factor CCAAT-enhancer binding protein β (C/EBPβ) (2), the cell cycle inhibitors (p21cip1 and p27kip1) (3, 4), and the steroidogenic enzyme P450scc (5). However, the regulation mechanisms and factors that are involved in the differentiation of granulosa cells to luteinize have been largely unidentified to date. Our preliminary microarray analyses, showing the up-regulation of ectodermal-neural cortex 1 (ENC1) gene by LH in cultured preovulatory follicles of pregnant mare’s serum gonadotropin (PMSG)-primed mice (data not shown), prompted us to hypothesize that ENC1 may act as a stimulator for differentiation during ovulation.

ENC1, named for its expression pattern in ectoderm and neural cortex and also called nuclear restricted protein/brain (NRP/B), is initially identified as a novel gene primarily and specifically expressed in neural tissue (6, 7). ENC1 belongs to a member of the kelch family of genes containing two major structural elements, BTB/POZ (Broad complex Tramtrack Bric-a-brac/Pox virus and Zinc finger in the N terminus), is initially identified as a novel gene primarily and specifically expressed in neural tissue (6, 7). ENC1 belongs to a member of the kelch family of genes containing two major structural elements, BTB/POZ (Broad complex Tramtrack Bric-a-brac/Pox virus and Zinc finger in the N terminus), and six copies of kelch repeats in the C terminus (6, 8). BTB/POZ domain

Abbreviations: BTB/POZ, Broad complex Tramtrack Bric-a-brac/Pox virus and Zinc finger in the N terminus; C/EBPβ, CCAAT-enhancer binding protein β; ENC1, ectodermal-neural cortex 1; F-actin, filamentous actin; G-actin, globular actin; hCG, human chorionic gonadotropin; PKA, protein kinase A; PKC, protein kinase C; PMSG, pregnant mare’s serum gonadotropin; SDS, sodium dodecyl sulfate; SSC, saline sodium; TPA, 2-O-tetradecanophorbol-13-acetate.
mediates both homo- and heterodimerization in vitro, which might be important for its interaction with BTB/POZ-containing proteins (9, 10). Kelch motifs have functional significance in binding actin, protein folding, or protein-protein interaction (8, 11, 12). The biological role of ENC1 in the regulation of cytoskeletal organization by binding to actin has been demonstrated in limited in vitro cellular systems including nerve cells (6, 7), differentiating adipose tissue (13), human brain tumor cells (14, 15), and hairy cell leukemia (16). Based on the present findings demonstrating the transient stimulation of ENC1 expression by the LH surge and its physical interaction with actin in rat preovulatory follicles, we propose that ENC1 plays a role as an actin-binding protein for cytoskeletal reorganization during the differentiation of granulosa cells in ovulation.

Materials and Methods

Hormones and reagents

Ovine LH was obtained from the National Hormone and Pituitary Distribution Program (National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA). PMSG, human chorionic gonadotropin (hCG), forskolin, and 2-O-tetradecanophorol-1-acetate (TPA) were purchased from Sigma Chemical Co. (St. Louis, MO). Chemical inhibitors including RO 31-8220 and Rp-cAMP were obtained from Calbiochem (La Jolla, CA).

Animals

Female rats of the Sprague Dawley strain were purchased from Dae-han Laboratories (Chungbuk, Korea). Animals were maintained and treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals with institutional animal care and use committee approval. They were housed in groups in a room with controlled temperature and photoperiod (10 h dark, 14 h light with lights on at 0600 h). The animals had ad libitum access to food and water. Immature (26-d-old) rats were injected with 10 IU PMSG sc to induce multiple growth of preovulatory follicles. Two days later, some rats received a single ip injection of 10 IU hCG to induce ovulation, and ovaries or granulosa cells of preovulatory follicles were obtained at different time intervals for analysis.

Culture of granulosa cells of preovulatory follicles

Ovaries were isolated and incubated in DMEM/Ham’s F-12 containing 0.5 m sucrose and 10 mM EGTA at 37 C for 30 min. Ovaries were flattened to one layer to easily identify preovulatory follicles before collection of granulosa cells of preovulatory follicles by the method of follicular puncture using 23-gauge needles under a dissection microscope as previously described (17). Cells were counted using trypan blue and incubated with actin, protein folding, or protein-protein interaction (8, 11, 12). The biological role of ENC1 in the regulation of cytoskeletal organization by binding to actin has been demonstrated in limited in vitro cellular systems including nerve cells (6, 7), differentiating adipose tissue (13), human brain tumor cells (14, 15), and hairy cell leukemia (16). Based on the present findings demonstrating the transient stimulation of ENC1 expression by the LH surge and its physical interaction with actin in rat preovulatory follicles, we propose that ENC1 plays a role as an actin-binding protein for cytoskeletal reorganization during the differentiation of granulosa cells in ovulation.

Western blot analysis

Whole-cell lysate was prepared from granulosa cells of preovulatory follicles by extracting proteins with a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS supplemented with protease inhibitors and phosphatases. After centrifugation at 13,600 g for 15 min, the supernatant was collected for the analysis. Protein concentrations were determined by using bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL). Proteins (50 μg) were separated by 12% polyacrylamide gel electrophoresis containing 0.1% SDS and transferred to nitrocellulose membrane. The nitrocellulose sheet was blocked with 5% nonfat dry milk in Tris-buffered saline. The blots were then washed and incubated with anti-ENC1 monoclonal antibody (BD Biosciences, San Diego, CA). The blots were also reacted with anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-α-tubulin monoclonal antibody (Sigma), or anti-histone H4 polyclonal antibody (Santa Cruz Biotechnology). The blots were developed using a peroxidase-conjugated secondary antibody, and proteins were visualized by the enhanced chemiluminescence system.

In situ hybridization analysis

Ovaries were fixed at 4 C for 6 h in 4% paraformaldehyde in PBS, followed by immersion in 0.5 m sucrose in PBS overnight. After fixation, ovaries were embedded in Tissue-Tek O.C.T. compound (Miles Scientific, Elkhart, IN), quickly frozen in liquid nitrogen, and stored at −80 C until sectioning. On each poly-l-lysin (Sigma Chemical Co.) coated microscope slide, four ovarian sections (14 μm thick) obtained from different rats were mounted and fixed in 4% paraformaldehyde in PBS, and thereby each experiment contained ovaries of four different rats. The hybridization procedure was essentially the same as previously described (18). In brief, sections were pretreated serially with 0.2% HCl, 2× SSC, pronase E (0.125 mg/ml; Sigma), 4% paraformaldehyde, and acetic anhydride in triethanolamine. Hybridization was carried out at 32–55 C overnight in the mixture containing 35S-labeled rat ENC1 cRNA probe (107 cpm/ml), 50% formamide, 0.3 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 1× Denhardt’s solution, 10% dextran sulfate, 1 μg/ml carrier tRNA, and 10 μg dithiothreitol. Posthybridization washing was performed under stringent conditions that included ribonuclease A (25 μg/ml) treatment at 37 C for 30 min and a final stringency of 0.1× SSC. Slides were dipped into NTB-2 emulsion (Eastman Kodak), exposed at 4 C, and developed after 1–2 wk. The slides were stained with hematoxylin and eosin and examined under the light microscope with bright-field and dark-field illumination.

Subcellular fractionation

Nuclear and cytosolic extracts were prepared from preovulatory granulosa cells obtained from PMSG/hCG-treated ovaries using the protocol described previously (19). After washing cells twice with cold PBS and centrifugation at 1000 × g for 2 min, pellets were resuspended in buffer A [10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and protease inhibitor cocktail] and incubated on ice for 10 min. After centrifugation at 1000 × g for 2 min, the supernatant was discarded and pellets were resuspended in five packed cell pellet volumes of buffer B. The cells were homogenized in a tight-fitting 7-ml Dounce homogenizer by 15–20 up-and-down strokes and centrifuged for 20 min at 25,000 × g in a TOLA120.2 rotor (Beckman, Palo Alto, CA). After centrifugation, the supernatant containing cytosolic fractions was carefully collected, and the pellet was mixed for obtaining nuclear fractions with 0.11 vol of buffer B [0.3 mM HEPES (pH 7.9), 1.4 mM KCl, 30 mM MgCl2, 1 mM DTT, and protease inhibitor cocktail] and centrifuged at 100,000 × g for 60 min at 4 C. The supernatant was carefully decanted, and the pellet was resuspended in 3 ml buffer C (20 mM HEPES, 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, and protease inhibitor cocktail) per 1 × 106 cells with a