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Identification of Genes Regulated by PKCζ during Ovulation in the Rat[1]

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Abstract

Our previous study demonstrates a rapid activation of atypical PKCζ by the ovulatory dose of LH/hCG. The present study was therefore designed to identify PKCζ regulated-genes in rat ovarian preovulatory granulosa cells. Preovulatory granulosa cells cultured in the presence of myristoylated PKCζ pseudosubstrate peptide were subjected to identify differentially expressed genes by using anneling control primer RT-PCR. As a result, among sixteen genes identified, six genes (testin, glypican-4, retrovirus SC1, connective growth factor, aminolevulinic acid synthase1 and serum- inducible kinase) were rapidly stimulated by hCG. Northern blot analysis demonstrated that all these genes were rapidly stimulated by hCG and declined thereafter. In situ hybridization analysis revealed the expression of these genes in granulosa cells of preovulatory follicles. The present study demonstrates time- and cell-specific expression of PKCζ-regulated genes, and may imply that these genes play a specific role(s) during LH-induced ovulation.

Key words: PKCζ, ovulation, preovulatory follicle, gonadotropin

INTRODUCTION

The preovulatory surge of LH is obligatory to trigger ovulation, a process by which preovulatory follicles rupture, release a fertilizable oocyte and undergo luteinization. Several studies have shown that the LH surge induces many genes associated with ovulation including the progesterone receptor, ADAMTS1, cyclooxygenase-2, growth differentiation factor-9 and CAAT enhancer binding protein beta (1). However, the precise signaling pathways that activate these ovulation-associated genes are largely unknown.

It has been indicated that low concentrations of LH preferentially activate adenylyl cyclase, whereas higher concentrations of the hormone also increase intracellular calcium and activate protein kinase C (PKC; 2). Our previous report demonstrates the activation of PKCζ by LH in preovulatory follicles (3). The present study was therefore designed to investigate genes regulated by PKCζ activation during ovulation in the rat.

MATERIALS AND METHODS

Animals

Female rats of the Sprague Dawley strain were purchased from Daehan 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. They were housed in groups in a room with controlled temperature and photoperiod (10-h dark, 14-h light, with lights on from 0600-2000 h). The animals had *ad libitum* access to food and water. Ovaries were collected from immature (26-day-old) rats at various times after treatment with 10 IU pregnant mare's serum gonadotropin (PMSG; Sigma) to induce multiple follicle growth. Some rats received a single ip injection of 10 IU human chorionic gonadotropin (hCG; Sigma) to induce ovulation

Anneling control primer (ACP) RT-PCR

To identify PKCζ - regulated genes preovulatory granulosa cells collected from PMSG-primed immature rat ovaries were cultured for 1.5 h in serum-free conditions under 95% air-5% CO_2 at 37 ℃ with LH in the presence of myristoylated PKCζ pseudosubstrate peptide or a similarly sized control peptide. Total RNA was subjected to perform (ACP) RT-PCR according to the manufacturer's instructions (Seegene, Seoul, South Korea). PCR products were cloned into pGEM-T easy vector (Promega, Madison, WI) and sequenced.

Northern blot analysis

Total RNA from was isolated using Tri Reagent solution. Twenty micrograms of total RNA were fractionated by electrophoresis on a 1% agarose gel containing formaldehyde and transferred to nylon membranes by capillary blotting with 10 × sodium citrate-sodium chloride (SSC). Hybridization was performed overnight at 42 ℃ with 32P-labeled rat complementary DNA (cDNA) probe. The signals were normalized to the 28S ribosomal RNA internal control.

In situ hybridization

Ovaries were fixed at 4 ℃ for 6 h in 4% paraformaldehyde in phosphate buffered saline (PBS), followed by immersion in 0.5 M sucrose in PBS overnight. Cryostat sections (14-μM thick) were mounted on poly-L-lysine (Sigma Chemical Co.)-coated microscope slides, fixed in 4% paraformaldehyde. The hybridization procedure was essentially the same as previously described using ^{35}S-labeled cRNA probe (4). After post-hybridization, the slides were stained with hematoxylin and eosin and examined under the light microscope with bright- and dark-field illumination.

RESULTS AND DISCUSSION

As a result of ACP RT-PCR, sixteen genes were differentially expressed by PKCζ activation and analyzed using the BLAST server. Particularly, six genes were rapidly stimulated by hCG (Fig. 1). Remaining genes were stimulated around the time of ovulation (data not shown).

Testin, known to hartbour a tumor suppressor gene, is predicted to encode a highly conserved protein of 421 amino acids containing three C-terminal LIM domain (5). Northern blot analysis showed that two different testin transcripts in size were detected in the ovary (Fig. 1). Treatment of PMSG-primed rats with hCG resulted in the rapid and transient stimulation of testin mRNA expression reaching a maximum stimulation 3 h after hCG treatment. *In situ* hybridization analysis revealed the expression of testin mRNA in the granulosa cells of preovulatory follicles (Fig. 2).

Glypicans are a family of cell surface heparan sulphate proteoglycans (HSPG), characterized by attachment to the cell surface by a glycosylphosphatidylinositol (GPI) anchor. Among six glypican isoforms, *glypican-4 (gpc4)* encodes a protein that has a C-terminal GPI anchor and several potential sites for attachment of heparan sulphate side chains (6). Glypican-4 mRNA levels were increased from 3 h to 24 h after gonadotropin treatment (Fig. 1). Glypican 4 mRNA was detected in theca cells of growing follicles as well as in interstitial cells in the ovaries of PMSG treated immature rat (Fig. 2). However, in ovaries of PMSG primed immature rats, followed by hCG stimulation for 6 h, glypican 4 mRNA was detected in granulosa cells of preovulatory follicles.

Retroviruses may be expressed in a cell-specific, ubiquitous, or limited manner (7). Retroviruse SC1 is expressed at high levels in rat granulosa cells and prepubertal sertoli cells (8). The present study demonstrated an increase in levels of ovarian retrovirus SC1 mRNA at 3 and 6 h after hCG administration (Fig. 1). The strong hybridization signal for SC1 mRNA was located to theca-interstitial cells layers of growing follicles in the ovaries of untreated immature rats (Fig. 2). Furthermore,

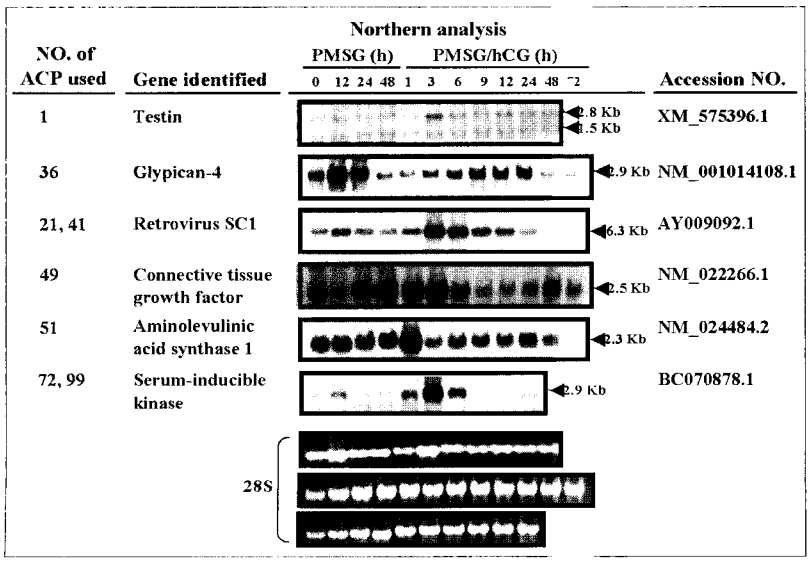

Fig. 1. Genes stimulated by PKCζ activation showing a rapid and transient increase by LH/hCG. Twenty micrograms of total RNA isolated from ovaries at the indicated time intervals after PMSG or PMSG/hCG stimulation were assayed for mRNA levels of each differentially expressed gene by Northern blotting using a rat cDNA probe. The estimated size of transcript was indicated. The expression of 28S ribosomal RNA was used as an internal standard. Each data are representative of two or three independently performed experiments.

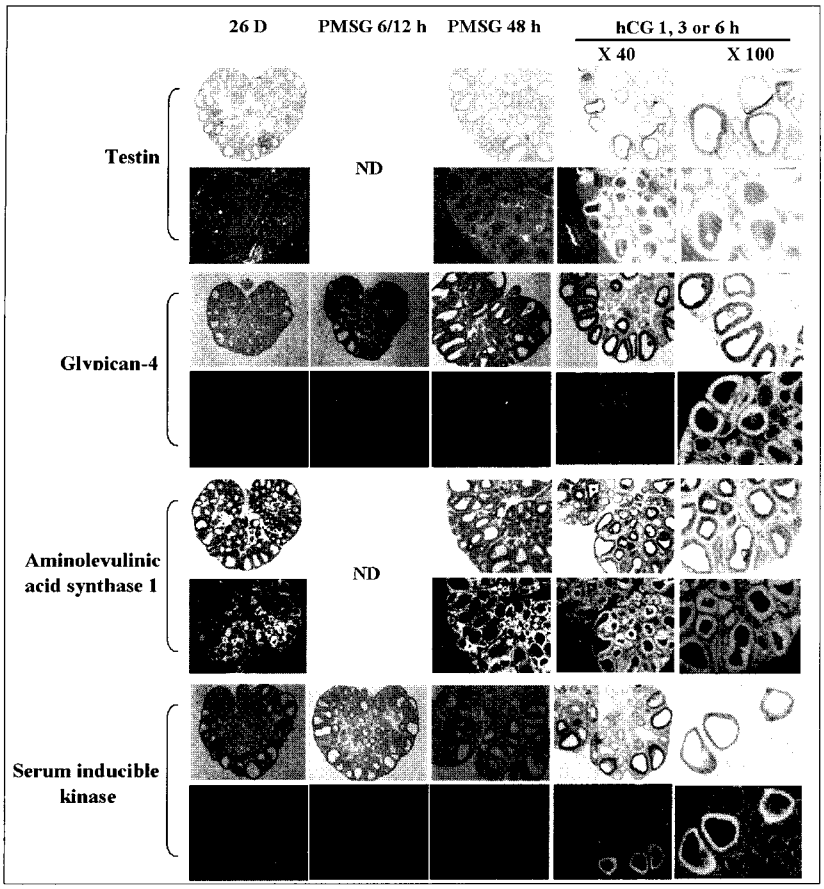

Fig. 2. Cellular localization of genes regulated by PKCζ activation in the immature rat ovary before and after gonadotropin treatment. Sections of ovaries from immature rats that were either untreated or treated with PMSG for 6/12, 48 h and subsequently treated with hCG for 1, 3, 6 h were hybridized with 35S-labeled rat cRNA probes. Photomicrographs were taken under bright- and dark-field illumination.

high expression of SC1 mRNA was localized to both granulosa cells of preovulatory follicles and the theca-interstitial layer at 3 h after hCG.

Aminolevulinic acid synthase1 (Alas1) is perhaps best known for its role in catalyzing the condensation of glysine and succinyl-CoA to yield 5-aminolevulinate, a universal precursor of tetrapyrrole compounds that function in a variety of reactions including the biogenesis of heme (9). Moderate levels of alas1 mRNA were detected in both immature untreated or PMSG-treated ovaries before hCG injection (Fig. 1). Treatment with hCG caused rapid and transient stimulation of alas1 mRNA expression, reaching a maximum stimulation within 1 h. Alas1 was detected in theca and interstitial cells of growing follicles and preovulatory follicles after PMSG treatments (Fig. 2). Interestingly, however, in ovaries of PMSG primed immature rats, followed by hCG stimulation for 1 h, alas1 mRNA was detected in granulosa cells of preovulatory follicles.

Serum-inducible kinase (Snk) bears a sequence homology with the polo-like kinases (10). Treatment of PMSG-primed rats with hCG resulted in the rapid and transient stimulation of snk mRNA levels (Fig. 1). In situ hybridization analysis revealed that snk signals were detected in theca cells and interstitial cells of growing follicles in ovaries obtained 6 h after PMSG treatment (Fig. 2). Interestingly, in ovaries of PMSG primed immature rats followed by hCG stimulation for 3 h, snk mRNA was detected in granulosa cells of preovulatory follicles but not of growing follicles.

In the present study, identification of PKCζ-regulated genes that are dramatically and transiently increased after hCG injection in granulosa cells of preovulatory follicles may imply a specific role of PKCζ signaling pathway during the ovulatory process. These PKCζ-regulated genes have generally been implicated in cell growth, survival, differentiation, and cytoskeleton reformation. Indeed, PKCζ pathway played a role in the survival of granulosa cells (data not shown). Therefore, it is plausible that stimulation of PKCζ by LH in preovulatory granulosa cells would be associated with the progression of granulosa cell differentiation during ovulation. The physiological functions and mechanism of these PKCζ-regulated genes need to be investigated.

References

1. Richards JS 1994 Hormonal control of gene expression in the ovary. Endocr Rev 15:725-751
2. Guderman T, Birnbaumer M, Birnbaumer L 1992 Evidence for dual coupling of the murine luteinizing hormone receptor to adenylyl cyclase and phosphoinositide breakdown and Ca++ mobilization. J Biol Chem 267:4479-4488
3. Park JI, Park HJ, Lee YI, Seo YM, Chun SY 2003. Regulation of NGFI-B expression during the ovulatory process. Mol Cel. Endocrinol 202:25-29
4. Lee J, Park H-J, Choi H-S, Kwon H-B, Arimura A, Lee B-J, Choi W-S, Chun S-Y 1999 Gonadotropin stimulation of pituitary adenylate cyclase-activating polypeptide (PACAP) messenger ribonucleic acid in the rat ovary and the role of PACAP as a follicle survival factor. Endocrinology 140:818-826
5. Tobias ES, Hurlstone AFL, MacKenzie E, McFarlane R, and Black DM 2001 The TES gene at 7q31.1 is methylated in tumours and encodes a novel growth-suppressing LIM domain protein. Oncogene 20:2844-2853
6. Watanabe K, Yamada H, and Yamaguchi Y 1995 K-glypican: a novel GPI-anchored heparan sulfate proteoglypican that is highly expressed in developing brain and kidney. J Cell Biol 130:1207-1218
7. Tomonaga K, and Coffin JM 1998 Structure and distribution of endogenous nenecotropic murine leukemia viruses in wild mice. J Virol 72:8289-8300
8. Anway MD, Johnston DS, Crawford D, and Griswold MD 2001 Identification of a novel retrovirus expressed in rat sertoli cells and granulosa cells. Biol Reprod 65: 1289-1296
9. Ferreira GC, and Gong J 1995 5-aminolevulinate synthase and the first step of heme biosynthesis. Int J Hematol 27:151-159
10. Ma S, Liu M, Yuan Y, and Erison RL 2003 The serum-inducible protein kinase snk is a G1 phase polo-like kinase that is inhibited by the calcium-and integrin-binding protein CIB. Mol Cancer Res 1:376-384