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Progesterone production is affected by unfolded protein response (UPR) signaling during the luteal phase in mice

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ABSTRACT

Aims: We examined whether the three unfolded protein response (UPR) signaling pathways, which are activated in response to endoplasmic reticulum (ER)-stress, are involved in progesterone production in the luteal cells of the corpus luteum (CL) during the mouse estrous cycle.
Main methods: The luteal phase of C57BL/6 female mice (8 weeks old) was divided into two stages: the functional stage (16, 24, and 48 h) and the regression stage (72 and 96 h). Western blotting and reverse transcription (RT)-PCR were performed to analyze UPR protein/gene expression levels in each stage. We investigated whether ER stress affects the progesterone production by using Tm (0.5 μg/g BW) or TUDCA (0.5 μg/g BW) through intra-peritoneal injection.
Key findings: Our results indicate that expressions of Grp78/Bip, p-eIF2α/ATF4, p50ATF6, and p-IRE1/sXBP1 induced by UPR activation were predominantly maintained in functional and early regression stages of the CL. Furthermore, the expression of p-JNK, CHOP, and cleaved caspase3 as ER-stress mediated apoptotic factors increased during the regression stage. Cleaved caspase3 levels increased in the late-regression stage after p-JNK and CHOP expression in the early-regression stage. Additionally, although progesterone secretion and levels of steroidogenic enzymes decreased following intra-peritoneal injection of Tunicamycin, an ER stress inducer, the expression of Grp78/Bip, p50ATF6, and CHOP dramatically increased.
Significance: These results suggest that the UPR signaling pathways activated in response to ER stress may play important roles in the regulation of the CL function. Furthermore, our findings enhance the understanding of the basic mechanisms affecting the CL life span.

Introduction

The corpus luteum (CL) is a transient endocrine gland that produces progesterone, which is required for pregnancy maintenance and estrous cycle regulation (Caligioni, 2009). After ovulation, a high level of progesterone blocks gonadotropin secretion and restricts follicular development as the CL develops. If the oocyte is not fertilized, CL regression is necessary for follicular growth, ovulation, and initiation of the estrous cycle (Setty and Mills, 1987).

Steroidogenic enzymes such as steroidogenic acute regulatory enzyme (StAR), p450 cholesterol side-chain cleavage enzyme (P450scc), and 3β-hydroxysteroid dehydrogenase (3β-HSD) are needed to produce progesterone in luteal cells of the CL, which are crucial for maintaining CL function (Black et al., 2005). The loss of capacity to produce progesterone followed by loss of luteal cell function leads to CL regression (Niswender et al., 2000). Therefore, we believe that steroidogenic enzyme synthesis in luteal cells of the CL play an important role in regulating the CL life span.

The endoplasmic reticulum (ER) is the site of protein synthesis and protein folding (Oyadomari and Mori, 2004). ER functional overload, including excessive protein synthesis and accumulation of misfolded and/or unfolded proteins in the ER lumen, results in ER stress through activation of the unfolded protein response (UPR) (Ito et al., 2009).

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The three major transducers of UPR are activating transcription factor 6 (ATF6), inositol-requiring enzyme1 (IRE1), and protein kinase-like ER kinase (PERK), which act as proximal sensors of ER stress (Jager et al., 2012). Under ER stress, sequestration of the ER chaperone protein Grp78/Bip by unfolded proteins activates these three sensors by inducing phosphorylation of IRE1 and PERK/eukaryotic translation initiation factor 2α (eIF2α), as well as by cleavage of ATF6 (Mori, 2000). The UPR is essentially cell-protective, but an excessive or prolonged ER stress can result in cell death through induction of ER stress-mediated apoptosis (Tay et al., 2012). ER stress-induced apoptosis is mediated by the transcriptional activation of the transcription factor CCAAT or enhancer binding protein (C/EBP)-homologous protein (CHOP), and activation of pro-apoptotic Jun N-terminal kinase (JNK) and caspase cascade through three UPR signaling pathways, which in turn promote apoptosis (Carambula et al., 2002).

ER stress has been mainly studied in active secretory cells (Kim et al., 2012). Luteal cells of the CL belong to the other type of endocrine secretory cells characterized by progesterone synthesis. However, the mechanism of UPR signaling regulation during steroidogenic enzyme expression in luteal cells of the CL and the effect of UPR signaling on CL life span are not fully understood. Furthermore, the correlation between ER stress and progesterone production in the mouse estrous cycle has not yet been investigated. Therefore, in this study, we determined whether the three UPR pathways (IRE1, PERK, and ATF6) are involved in the functional stage of the CL and whether ER stress-mediated apoptosis occurs during the regression stage in mouse CL tissue. We also investigated whether ER stress inducers or inhibitors affect not only steroidogenic enzymes expression but also progesterone production in female mice.

Materials and methods

Animal treatment and CL collection from ovaries

Female mice C57BL/6 (8 weeks old) were purchased from Hyochang Bio-Science (Daegu, Korea) and maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, South Korea). The estrous cycles of female mice can be synchronized by hormone priming with pregnant mare serum gonadotropin (PMSG; Sigma, St. Louis, MO) and human chorionic gonadotropin (hCG; Sigma) via intra-peritoneal (i.p.) injection. One week later, female mice received i.p. injection with 5 IU PMSG per mouse followed by 5 IU hCG injection after 48 h. This treatment led to ovulation after 12 h, which was followed by CL development. Based on the progesterone concentration and ovarian morphological features, the luteal phase was divided in to two stages: the functional stage (16, 24, and 48 h) and the regression stage (72 and 96 h), as previously described (Lee et al., 2012). The beginning of CL formation was considered to be at 16 h after PMSG/hCG injection (Supplementary Fig. 2). Mice were killed, blood samples were collected, and the ovaries were removed at the indicated time intervals. CL tissues were collected from the ovaries under a dissecting microscope and immediately frozen at −70 °C until use. All experiments were performed in triplicate, and each experiment was independently analyzed.

Administration of Tm and TUDCA in female mice

C57BL/6 female mice (8 weeks old) were administered Tunicamycin (Tm; 0.5 μg/g body weight; Calbiochem, La Jolla, CA) as an ER stress inducer, or Tauroursodeoxycholic acid (TUDCA;0.5 μg/g BW; Calbiochem) was administered as an ER stress inhibitor by i.p. injection at the indicated times (16 h). The controls were administered saline by i.p. injection. After administration, the mice were sacrificed at the indicated times, and blood and CL tissues were collected.

Progesterone assay by EIA

To measure progesterone concentration, blood samples were collected from the orbital sinuses of female mice at the indicated times after injection. We separated the serum by centrifugation at 8000 *g* for 15 min at 4 °C and then stored it at −20 °C until progesterone assays were conducted. Progesterone production was assessed according to the manufacturer's instructions by using a progesterone enzyme immunoassay (EIA) kit (ALPCO, Salem, NH). Progesterone concentration of each sample was calculated using the standard graph and expressed in ng/ml.

RNA extraction and reverse transcription (RT)-PCR

Total RNA was isolated from the CL tissues of mouse ovaries according to the manufacturer's instructions using a TRIzol reagent (Invitrogen, Carlsbad, CA). RNA concentration and purity were measured with a NanoDrop spectrophotometer (ACTGene, Piscataway, NJ). Subsequently, 1 μg/μl of total RNA and AccuPower® RT-PCR Premix (Bioneer Inc., Daejeon, South Korea) was used to synthesize cDNA. Primers specific for the sequences of interest (Table 1) were designed using the NCBI database. PCR was carried out at 95 °C for 5 min, followed by 30 cycles comprising the following steps: 95 °C for 30 s, 55–60 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min. The PCR products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide (EtBr), and photographed under UV illumination. Band intensities were quantified using the ImageJ software (National Institutes of Health, MD).

Detection of XBP1 splicing by RT-PCR

XBP1 RNA splicing was detected by standard RT-PCR using RNA templates isolated from the CL tissues. First-strand cDNA was synthesized from 1 μg of each total RNA sample using oligo (dT) primers and AccuPower® RT-PCR (Bioneer). PCR was carried out using 2× PCR Premix (Enzynomics, Daejeon, South Korea) containing specific primers (Table 1). The PCR products were digested with *Pst*I (NEB, MA, USA) for 90 min at 37 °C and then separated by electrophoresis in 2% agarose gels.

Protein extraction and Western blot assays

CL tissue lysates were prepared in ice-cold PRO-PREP protein lysis buffer (iNtRON, Daejeon, South Korea). The protein concentration of each sample was estimated using a Bradford dye-binding assay. Aliquots of the proteins (30 μg) were separated by SDS-PAGE in 12% gels. After electrophoresis, the separated proteins were transferred onto nitrocellulose membranes (Pall Corporation, Port Washington, NY). After blocking with 5% non-fat milk in Tris-buffered saline (TBS) with 0.1% Tween 20 at 4 °C with shaking, the membranes were incubated with the following antibodies: anti-GADD153/CHOP, anti-CREB2/

Table 1
Primer sequence for reverse transcription PCR 3β-HSD: 3β-hydroxysteroid dehydrogenase, StAR: steroidogenesis acute regulatory, P450scc: cytochrome P450 side-chain cleavage, XBP1:X-box binding protein 1, Tm: melting temperature, For: forward, Rev: reverse.

Target	Primer	Sequence reported 5′–3	Tm °C	Length (bp)
3β-HSD	FOR	ACTGCAGGAGGTCAGAGCT	55	401
NM_008293.3	REV	GCCAGTAACACACAGAATACC		
XBP1 AF027963.3	FOR	AAACAGAGTAGCAGCGCAGACTGC	65	743
	REV	TCCTTCTGGGTAGACTTCTGGGAG		
StAR NM_011485.4	FOR	GAAAAGACACGGTCATCACT	56	262
	REV	CCGTGTCTTTTCCAATCCTC		
P450scc (Cyp11a1)	FOR	GCTGGAAGGTGTAGCTCAGG	57	224
AF195119.1	REV	CACTGGTGTGGAACATCTGG		
GAPDH BC145810.1	FOR	ACCACAGTCCATGCCATCAC	55	452
	REV	TCCACCACCCTGTTGCTGTA		