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Unfolding protein response signaling is involved in development, maintenance, and regression of the corpus luteum during the bovine estrous cycle

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ABSTRACT

The corpus luteum (CL) is a transient endocrine organ. Development, maintenance, and regression of CL are effectively controlled by dynamic changes in gene expression. However, it is unknown what types of gene are affected during the CL life span of the estrous cycle in bovine. Here, we determined whether unfolded protein response (UPR) signaling via eIF2α/ATF4/GADD34, p90ATF6/p50ATF6, and IRE1/XBP1, which is a cellular stress response associated with the endoplasmic reticulum (ER), is involved in the bovine CL life span. Our results indicated that expression of Grp78/Bip, the master UPR regulator, was increased during the maintenance stage and rapidly decreased at the regression stage. Additionally, UPR signaling pathways genes were found to be involved in luteal phase progression during the estrous cycle. Our findings suggested that Grp78/Bip, ATF6, and XBP1 act as ER chaperones for initiating CL development and maintaining the CL. In addition, we investigated whether ER stress-mediated apoptosis is occurred through three UPR signaling pathways in CL regression stage. Interestingly, pIRE1 and CHOP were found to be involved in both the adaptive response and ER stress-mediated apoptosis. During the CL regression stage, increased expression of pJNK and CHOP, two components of ER stress-mediated apoptotic cascades, occurred before increased level of cleaved caspase 3 were observed. The present investigation was performed to identify a functional link between UPR signaling and CL life span during the bovine estrous cycle. Taken together, results from this study demonstrated that UPR protein/gene expression levels were different at various stages of the bovine CL life span. Variations in the expression of these protein/genes may play important roles in luteal stage progression during the estrous cycle.

1. Introduction

The corpus luteum (CL) is a transient endocrine organ. The main function of this organ is to produce progesterone (P4), which is necessary for pregnancy maintenance and estrous cycle regulation [1]. Rapid development of the CL occurs after ovulation [2], and is accompanied by the proliferation and differentiation of granulosa cells into luteal cells [3]. If pregnancy does not occur, the CL regresses within a few days and a new ovulation cycle begins [2].

The functional capacity of the CL to produce progesterone may also be associated with regulation of the CL life span. Luteal cells of the CL are secretory cells. Three steroidogenic enzymes are required for progesterone production by the luteal cells: steroidogenic acute regulatory protein (StAR), the p450 cholesterol

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side-chain cleavage enzyme (P450scc), and 3β-hydroxysteroid dehydrogenase (3β-HSD) [4]. Therefore, these steroidogenic enzymes are consistently needed for progesterone synthesis. Thus, it is likely that the ER of luteal cells is also involved in the synthesis of steroidogenic enzymes that regulate the CL life span.

The endoplasmic reticulum (ER) plays a central role in protein synthesis and folding [5,6]. ER functional overload, including excessive protein synthesis and accumulation of unfolded proteins in the ER lumen, results in ER stress [6]. For survival and adaption, cells have developed the unfolded protein response (UPR), a self-protective mechanism against ER stress [7]. The UPR of mammalian cells is mediated by three ER transmembrane proteins that act as proximal sensors of ER stress [6,8]: activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1), and protein kinase-like ER kinase (PERK). In the presence of ER stress, sequestration of the ER chaperone protein Grp78/Bip by unfolded proteins activates these three sensors by inducing the phosphorylation of IRE1 and PERK/eukaryotic translation initiation factor 2α (eIF2α), and cleavage of ATF6 [9]. The UPR is fundamentally cyto-protective, but an excessive or prolonged UPR expression can result in cell death predominantly through the induction of ER stress-mediated apoptosis [10]. Prolonged ER stress induces the activation of the pro-apoptotic C/EBP homologous protein (CHOP), Jun N-terminal kinase (JNK), and caspases through three UPR pathways, which in turn promotes apoptosis [11]. However, it is not clear whether UPR signals affect the CL life span.

In a previous study, we demonstrated that the UPR plays important roles in steroidogenic enzyme expression by modulating the ATF6 pathway as well as ER stress-mediated apoptosis in human chorionic gonadotropin (hCG)-stimulated Leydig cells [12]. Therefore, we hypothesized that the UPR may significantly influence the CL life span by regulating steroidogenic enzyme expression in bovine CL. The goal of the present study was to investigate differences in the expression of UPR-associated genes at the protein and RNA level in bovine CL tissues according to the CL life span stage.

2. Materials and methods

2.1. Collection of CLs from ovarian tissues

Ovaries were obtained during the estrous cycle from non-pregnant cows at a local abattoir (Kimhae, Kyungnam, Korea) transported to the laboratory [13]. CL tissues were removed from the ovaries in the laboratory (Supplementary Fig. 1). The luteal phase was divided into three main groups according to macroscopic appearance of the CL tissues as previously described [14,15]. As shown Fig. 1A, the early stage of the CL is characterized by the formation of reddish brown soft tissue (development; Early 1 and 2). CL tissues of the mid stage are tan and orange, and large and hard (maintenance; Mid 1–3). CL tissues of the late stage are completely orange to light yellow and small in size (regression; Late 1).

And each group was further subdivided in the following manner: development [Early 1 (2–4 d after ovulation) and 2 (5–7 d after ovulation)], maintenance [Mid 1 (8–11 d post-ovulation), 2 (12–14 d post-ovulation), and 3 (15–17 d post-ovulation)], and regression [Late 1 (18–20 d post-ovulation)]. For one independent experiment, we obtained CL tissue sample from at least seven ovaries in each luteal stage ($n = 7$ ovaries per stage).

2.2. RNA extraction and reverse transcription PCR

Total RNA was isolated from each individual CL tissue sample using TRIzol reagent (Invitrogen, CA) according to the manufacturer's instructions. Each cDNA was synthesized from the aliquots (1 μg/μl) of total RNA with AccuPower® RT-PCR Premix (Bioneer, Korea). PCR was carried out using AccuPower® PCR Premix (Bioneer) containing specific primers for steroidogenic enzymes (Table 1). The PCR products were separated by electrophoresis in 2% gels. Band intensities were quantified with ImageJ software (NIH, Bethesda, MD).

2.3. Detection of XBP1 splicing by RT-PCR

The cDNA was synthesized from 1 μg of each total RNA using oligo(dT) primers and AccuPower® RT-PCR (Bioneer, Korea). PCR was performed using 2× PCR Premix (Enzynomics, Korea) containing specific primers (Table 1) for splicing XBP-1. The PCR products were digested with Pst1 and then separated by electrophoresis in 2% agarose gels.

2.4. Protein extraction and Western blotting

Total proteins were extracted from CL in ice-cold PRO-PREP protein lysis buffer (iNtRON, Korea). CLs lysate were separated by SDS–PAGE in 12% gels. After electrophoresis, the separated proteins were transferred onto nitrocellulose membranes (Pall Life sciences, NY). After blocking, the membranes were incubated with anti-GADD153, anti-GADD34, anti-CREB2/ATF4, anti-p90ATF6, anti-3β-HSD (Santa Cruz Biotechnology, CA), anti-Grp78/Bip, anti-IRE1, anti-eIF2a, anti-phospho-eIF2a, anti-SAPK/JNK, anti-phospho-SAPK/JNK, anti-cleaved caspase 3 (Cell Signaling, MA), anti-phospho-IRE1 (Abcam, MA), and anti-p50ATF6 (obtained from Dr. In Kyu Lee) antibodies. The membranes were then incubated with a secondary HRP-conjugated anti-goat/mouse/rabbit IgG (Thermo, Scientific, MA). Antibody binding was detected using an ECL kit (Advansta, CA).

2.5. Statistical analysis

All data were analyzed using a one-way ANOVA followed by Dunnett's multiple comparison tests. All calculations were performed using the GraphPad Prism 5.0 software package (San Diego, CA). Differences were considered significant at $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

3. Results

3.1. Steroidogenic gene expression in the CL is altered according to the luteal phase of the bovine estrous cycle

Western blotting results showed that the expression of 3β-HSD protein was increased 2.2-fold by Mid 1 ($p < 0.05$) compared to the Early 1 stage (Fig. 1E). Moreover, 3β-HSD protein levels decreased thereafter and almost disappeared during the Late 1 stage ($p < 0.001$ compared to the Mid 1). In addition, expression patterns of StAR, P450ssc and 3β-HSD mRNA as steroidogenic enzymes involved in progesterone synthesis were similar to those of 3β-HSD protein expression (Fig. 1B–D). Based on these results, we determined that the CL tissues could be used for the subsequent experiments.

3.2. UPR signaling is involved in luteal phase progression during the estrous cycle

To confirm whether the three main UPR pathways affect the CL life span from development to regression, we measured the expression levels of eIF2α/ATF4/GADD34, p90ATF6/p50ATF6, and IRE1/XBP1. Initially, we observed that levels of Grp78/Bip protein as a major ER stress marker were significantly increased and maintained until Mid 3 ($p < 0.05$; Fig. 2A), and significantly de-