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Hypoxia regulates cell proliferation and steroidogenesis through protein kinase A signaling in bovine corpus luteum

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ABSTRACT

Hypoxia is an important physiological process which ensures corpus luteum (CL) formation and development, thus playing an important role in steroidogenesis. Recent studies have shown that CL develops in an analogous to tumorigenesis by accumulation of hypoxia-inducible factor-1 alpha subunit (HIF1A) in response to hypoxia. To investigate the relationship among hypoxia, steroidogenesis, and cell proliferation during CL lifespan, histological and steroidogenic analyses of CL were performed at various CL stages in non-pregnant Holstein. Also, the hypoxia-mediated steroidogenesis and cell proliferation were studied in vitro with both primary luteal and luteinized granulosa cells. Our results showed that progesterone (P_4) concentration increased with the upregulation of steroidogenic protein including steroidogenic acute regulatory protein (STAR) and CYP11A1 (P450scc) in the middle luteal stage. On the other hand, the cell proliferation- or hypoxia-associated proteins were upregulated in the early stage, including the proliferating cell nuclear antigen (PCNA), vascular endothelial growth factor A (VEGFA), HIF1A, and aryl hydrocarbon receptor nuclear translocator (ARNT). In primary culture, phospho-protein kinase A (p-PKA) was downregulated, as were P_4 secretion and steroidogenic proteins both under oxygen-conditioned hypoxia in luteal cells and cobalt chloride-induced hypoxia in luteinized granulosa cells. However, under the treatment of hypoxia, PCNA, which was downregulated in luteal cells, was upregulated together with HIF1A and VEGFA in luteinized granulosa cells. Taken together, present study suggested that hypoxia downregulated steroidogenesis through PKA signaling and that the hypoxia-regulated cell proliferation could be activated during CL formation.

1. Introduction

In mammals, corpus luteum (CL) is a dynamic endocrine gland during the estrus cycle (Devoto et al., 2002). While receiving the luteinizing hormone (LH) surge as the luteotrophic signal from adenohypophysis, the ovulation of preovulatory follicles and CL formation are triggered (Richards et al., 2002). During the latter process, progesterone (P_4)-producing cells are luteinized from the theca internal and granulosa cells of the follicle wall (Devoto et al., 2002; Robker et al., 2009). And the newly formed CL is also characterized by a series of cellular activities, including the rapid angiogenesis and growth of luteal size (Koga et al., 2000).

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The secretion of progesterone serving as the major functional approach of CL, steroidogenesis has been well studied in bovine (Stocco, 2001; Stocco et al., 2007). In luteal cells, cholesterol, which has been regarded as the original source of progesterone production, is transported from cytoplasm into mitochondrial through steroidogenic acute regulatory protein (STAR) (Stocco, 2001). And the enzyme CYP11A1 (also called P450scc) in mitochondrial conducts the side chain cleavage reaction to generate pregnenolone (P_5) (Simpson, 1979). Progesterone is then synthesized through the reaction of 3β-hydroxysteroid dehydrogenase (HSD3B) at endoplasmic reticulum (ER) (Simard et al., 2005). While gonadotropins (LH or hCG) upregulate steroidgenic genes and stimulate the progesterone synthesis, such an action can be controlled through cAMP-Protein kinase A (PKA) pathway (Oonk et al., 1989; Dyson et al., 2009). Recently, it has come to light that hypoxia could be involved in the angiogenesis and steroidogenesis during CL development (Koga et al., 2000; Klipper et al., 2010; Nishimura and Okuda, 2010). Hypoxia-inducible factor-1 (HIF1) is a heterodimeric transcription factor, which is composed of an oxygen-regulated alpha (HIF1A) subunit and a constitutive-expressed aryl hydrocarbon receptor nuclear translocator (ARNT) (Wang et al., 1995; Semenza, 2003). Under hypoxia, the inhibition of oxygen-dependent-degradation pathway of HIF1A leads to its accumulation, causing itself to be bound to ARNT (Semenza, 2010). Composed HIF1 then acts as a transcription factor to regulate downstream genes including the vascular endothelial growth factor A (VEGFA), a marker of angiogenesis which might contribute to the formation of CL (Semenza, 2003; Nishimura and Okuda, 2010). And the relationship between hypoxia and steroidogenesis has been studied in luteal cells at various stages (Nishimura et al., 2006, 2008; Nishimura and Okuda, 2010). The investigation of various cell responses has suggested that hypoxia may play a variety of roles during the lifespan of CL (Nishimura and Okuda, 2010). However, the detailed responses of steroidogenic genes to hypoxia at protein level, and their situational reflections in vivo remain to be identified. In addition, the responses to hypoxia in steroidogenesis and proliferation of steroidogenic cells in earlier stages call for further study.

Thus, present study was designed to investigate the effects of hypoxia on steroidogenesis in primary luteal cells and luteinized granulosa cells during the lifespan of bovine CL. Based on our hypothesis that HIF1A might play a role in angiogenesis and cell proliferation in CL, we investigated the expression and localization of steroidogenic and hypoxia-related proteins in bovine CL at various stages. And the studies on the effects of hypoxia on the gene expression profiles of steroidogenesis and cell proliferation were performed using middle stage luteal cells and luteinized granulosa cells.

2. Materials and methods

2.1. Collection of ovaries

Non-pregnant Holstein cow ovaries containing follicle or CL at various stages were collected at a local abattoir within 10 min of exsanguination. CLs were classified into three groups (early, middle, and late stages) based upon published literature (Miyamoto et al., 2000). All experimental protocols were approved by the Animal Care and Use Committee, College of Medicine, National Taiwan University. Individual CLs were bisected; one-half were frozen and the other half fixed in 10% (v/v) neutral buffered formalin for later analysis. For cell culture experiments, ovaries with middle luteal phase CLs and the preovulatory follicles were collected in ice cold phosphate-buffered saline (PBS) and transported to the laboratory for subsequent cell isolation (Miyamoto et al., 2000; Pauli et al., 2005; Duncan et al., 2008).

2.2. Immunohistochemistry (IHC) analysis

To evaluate the expression and location of steroidogenic proteins (STAR, CYP11A1 and HSD3B), specific antibodies generated previously were applied for IHC detection (Chin et al., 2008; Chiu et al., 2008). For the analysis of angiogenic proteins, specific primary antibodies were purchased from Thermo Scientific: HIF1A (HIF-1alpha Ab-4; MS-1164), ARNT (HIF-1beta Ab-1; MS-1165), VEGFA (Vascular Endothelial Growth Factor Ab-8; RB-1678), and PCNA (PCNA Ab-1; MS-106). The fixed (in 10% formalin solution overnight) bovine CL ($n = 5–8$ per luteal stage) tissues were embedded in paraffin, and sectioned (5-μm-thick). Tissue sections were then mounted on poly-L-lysine-coated glass slides. Following deparaffinization in xylene, tissue sections were rehydrated by passing through descending concentrations of ethanol and finally washed in Tris-buffered saline (TBS; 2 mM Tris–HCl, 15 mM NaCl, pH 7.6) at room temperature. Retrieval of antigen-binding sites was achieved with the incubation of citrate buffer (10 mM; pH 6.0) heated in microwave oven (100 W, 5 min) thrice. After quenching endogenous peroxidase activity with 1% H_2O_2 in PBS (pH 7.2) for 10 min, the sections were rinsed thrice (5 min each) in PBS. The slides were incubated in 0.02% bovine serum albumin (BSA) for 30 min to reduce non-specific binding of antibodies, followed by the incubation of primary antibodies for 24 h at 4 °C in a moist chamber, while the negative controls for these antibodies were established using bovine serum. After incubation with the primary antibody, the sections were rinsed and incubated with biotinylated horse anti-mouse/anti-rabbit secondary antibodies for 30 min in a moist chamber. The sections were rinsed again prior to incubation with an avidin–biotin–horseradish peroxidase complex (ABC-Method; Vectastain Universal ELITE ABC Kit; Vector Laboratories, Burlington, Ontario, Canada) for 45 min according to the manufacturer's protocol. After additional rinsing, slides were incubated for 10 min at room temperature with diaminobenzidine (DAB) for visualization of the immunostaining. Finally, the slides were rinsed in distilled water thrice (5 min each), counterstained with hematoxylin for 30 s, and rinsed in tap water prior to mounting with Kaiser's Gelatin (Merck; Darmstadt, Germany). Slides were observed under Axioskop 40 microscope (Carl Zeiss, Göttingen, Germany), equipped with digital camera (PowerShot A620, Canon, Tokyo, Japan). Luteal cells stained positive for STAR, CYP11A1, HSD3B,