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The newly formed corpora lutea of normal cycling rats exhibit drastic changes in steroidogenic and luteolytic gene expressions

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ABSTRACT

In normal estrous cycling rats, corpora lutea (CL) regress over several cycles; however, the period during which they secrete progesterone (P4) is strictly limited. In the present study, we clarified the function of CL in normal cycling rats. We especially focused on expression levels of four steroidogenic and two luteolytic genes in the two different populations of the CL (new and old CL) at each estrous stage. The ovaries of female rats at each estrous cycle were collected, and new and old CL were separated with laser microdissection and analyzed for mRNA expression. In the new CL, the expressions of scavenger receptor class B type I (*SR-BI*), steroidogenic acute regulatory protein (*StAR*), and P450 cholesterol side-chain cleavage (*P450scc*) mRNA reached their highest levels at metestrus, and 3β-hydroxysteroid dehydrogenase (3β-*HSD*) mRNA gradually increased from estrus to diestrus. Meanwhile, 20α-hydroxysteroid dehydrogenase (20α-*HSD*) and prostaglandin F2 alpha receptor (*PGF2α-R*) mRNA levels were remarkably low from estrus to metestrus and gradually increased thereafter. These gene levels in new CL corresponded to serum P4 levels during the estrous cycle. In the old CL, all steroidogenic and luteolytic gene levels were consistently high throughout the estrous cycle. These results provide clear evidence that new CL at metestrus have strong steroidogenic activity and through inhibition of luteolysis, maintain P4 production in normal cycling rats. The elevation of 20α-HSD and PGF2α-R levels in new CL at diestrus may be a trigger of functional luteolysis.

1. Introduction

Numerous drugs and chemicals that have been tested in experimental animals have been found to interfere with reproductive function in the female (Yuan and Foley, 2002). These chemicals, which usually target the ovaries, are commonly referred to as ovarian toxicants and frequently cause disturbances in estrous cyclicity in rodents. Other chemicals may act by altering the normal morphology of the reproductive tract (Yoshida et al., 2009). For instance, 4-vinyl-cyclohexene diepoxide (VCD) destroys oocytes and induces the decrease of small follicles (Ito et al., 2009), and ethylene glycol monomethyl ether (EGME) stimulates luteal progesterone (P4) secretion and induces luteal hypertrophy (Dodo et al., 2009). The ovary has two distinct functional components required for estrous cyclicity, the corpora lutea (CL) and the follicles. Understanding the morphology and function of these structures is a prerequisite for understanding the mechanism of ovarian toxicants that disrupt the estrous cycle. The rat estrous cycle is characterized by cyclic variation in P4 levels. There are two discrete periods in the estrous cycle during which P4 is increased. The first occurs in the afternoon of proestrus and the second during the metestrus to diestrus stages (Smith et al., 1975; Tebar et al., 1995). The preovulatory P4 is secreted during proestrus by the Graafian follicles in an luteinizing hormone (LH)-dependent manner. In metestrus and diestrus, secretion is from the CL in an LH-independent manner. The luteal secretion of P4 during the metestrus to diestrus stages begins to rise in the morning of metestrus, reaches peak values by midnight of metestrus, and falls to basal levels thereafter as a result of luteolysis (Kaneko et al., 1986). This drop-off in P4 is considered the beginning of the functional regression of the CL in the normal rat estrous cycle. Additionally, prolactin (PRL) has a crucial role in luteal P4 secretion and structural luteolysis (Stocco et al., 2007).

The P4 biosynthesis in the CL is divided into the following two steps: the uptake, synthesis, and transport of cholesterol, and the processing of cholesterol to P4. Cholesterol is preferentially yielded from circulatory high- and low-density lipoproteins (HDL and LDL);

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and HDL is the main source of cholesterol for CL in rodents (Bruot et al., 1982; Schuler et al., 1981). Scavenger receptor class B type I (SR-BI) is now considered as the authentic HDL receptor mediating the selective uptake of HDL-derived cholesterol ester (Acton et al., 1996). After uptake, the cholesterol esters are transported to the outer mitochondrial membrane and then to the inner membrane by several proteins including steroidogenic acute regulatory protein (StAR) (Stocco et al., 2001). Once cholesterol reaches the inner mitochondrial membrane, its transformation into P4 begins. In this step, mitochondrial P450 cholesterol side-chain cleavage (P450scc) (Oonk et al., 1989) and 3β-hydroxysteroid dehydrogenase (3β-HSD) which are located in the smooth endoplasmic reticulum (Peng et al., 2002) play principal roles.

The P4 secretion from the CL in rodents is regulated by the balance between synthesis and catabolism. Briefly, it depends not only on the amount of P4 synthesized by the luteal cells but also on the expression of the enzyme 20α-hydroxysteroid dehydrogenase (20α-HSD) that catabolizes P4 into the inactive progestin, 20α-dihydroprogesterone (20α-DHP). Once 20α-HSD becomes expressed in the CL, P4 secretion declines and 20α-DHP becomes the major steroid secreted by luteal cells (Stocco et al., 2000).

In rodents, the decrease in P4 is an index of the functional regression of the CL. The structural regression occurs after the initial decline in P4 output and is morphologically observed as luteal cell apoptosis (Stocco et al., 2007). In the functional regression, several factors including prostaglandin F2 alpha (PGF2α) and LH have been implicated in the downregulation of luteal P4 production (Pharriss and Wyngarden, 1969; Plas-Roser et al., 1988). Meanwhile, several signals including PRL, PGF2α, tumor necrosis factor-alpha (TNFα), and Fas ligand (FasL) have been indicated in the induction of cell death required for the structural regression of the CL (Gaytan et al., 2000; Roughton et al., 1999; Stocco et al., 2007; Yadav et al., 2005).

There are two main types of CL: those which are newly formed by the current ovulation (new CL) and CL remaining from prior estrous cycles (old CL) (Bowen and Keyes, 2000). New and old CL are morphologically distinguishable at each estrous stage, and new CL drastically change their morphology during the estrous cycle (Yoshida et al., 2009).

As mentioned above, the CL in cycling rats secrete P4 for a limited period prior to undergoing functional luteolysis a few days after being formed. It is likely that both new and old CL are essential to estrous cycle regulation. Therefore, it is important to analyze normal functional changes of steroidogenesis in each type of CL across the estrous cycle in order to understand how they may be affected by ovarian toxicants. The expression of steroidogenic and luteolytic factors across the estrous cycle has been partially elucidated (Peluffo et al., 2006; Slot et al., 2006; Takahashi et al., 1995); however, little is known about the transitions in gene expression that occur in new and old CL across the estrous cycle. In the present study, we investigated the transitions in luteal gene expression and steroidogenesis in each rat estrous stage to identify the potential targets of ovarian toxicants. We separated new CL from old CL using laser microdissection (LMD). We focused on four steroidogenic genes: *SR-BI*, *StAR*, *P450scc*, and *3β-HSD*, and two luteolytic genes: *20α-HSD* and PGF2α receptor (*PGF2α-R*). Additionally, immunohistochemical features of P450scc and 3β-HSD in both types of CL were also examined.

2. Materials and methods

2.1. Animals

Female 6-week-old Sprague–Dawley (Crl:CD) rats were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). They were housed in plastic cages (3 or 4 animals/cage) maintained at 23–25 °C and a relative humidity of 50–60% with a 12-h light cycle. Commercial rodent chow (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water were available *ad libitum* throughout the experiment. The animal protocols were reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Estrous cyclicity was monitored by daily vaginal smears. When the rats reached 10 weeks of age, they were euthanized by decapitation at each of the estrous stages (estrus, metestrus, diestrus, and proestrus: 6–7 rats per group) between 10:00 and 12:00 AM. These estrous stages were also confirmed by microscopic examination of the vagina and uterus. For LMD, the left ovaries were rapidly removed, embedded in OCT compound, and frozen with liquid nitrogen. The right ovaries were fixed in 4% paraformaldehyde for one day, and routinely processed with hematoxylin and eosin (HE) and immunohistochemical stains.

2.2. Laser microdissection of new or old corpora lutea in each estrous stage

The OCT-embedded frozen ovaries were sectioned into 10 μm slices onto membrane-based laser microdissection slides (Leica Microsystems, Wetzlar, Germany) and fixed in 70% ethanol for 1 min. The sections were then hydrated in diethylpyrocarbonate (DEPC)-treated water for 10 s, stained with toluidine blue for 30 s, washed in DEPC water for 30 s, dehydrated by dipping sequentially in 70, 95, and 100% ethanol and then air dried. New CL (CL which are newly formed by the current ovulation) and old CL (CL remaining from prior estrous cycles) were visualized and captured using a Leica LMD6000 laser microdissection system (Leica Microsystems) (Sakurada et al., 2006).

2.3. Extraction of total RNA and reverse transcription

Laser-captured tissues were pooled in lysis buffer and RNA was extracted with the RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Residual genomic DNA was removed by an on-column RNase-free DNase Set (Qiagen) during RNA purification. The RNA was then precipitated with 14 μl ddH_2O, checked for concentration and purity using the spectrophotometer (NanoDrop ND-1000, Thermo Fischer Scientific Inc., Waltham, MA, USA), and stored at −80 °C until analysis. For cDNA synthesis, reverse transcription (RT) was performed with the Sensiscript RT Kit (Qiagen) using random primers in a 20 μl final volume following the manufacturer's instructions.

2.4. Real-time quantitative PCR

Messenger RNA levels were analyzed using an ABI Prism 7900 Sequence Detection System and TaqMan® gene expression assays (Applied Biosystems, Foster City, CA, USA) for *SR-BI*, *StAR*, *P450scc*, *3β-HSD*, *20α-HSD*, *PGF2α-R*, and hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) (Table 1). The PCR cycling conditions included an initial denaturation at 95 °C for 20 s followed by 50 cycles at 95 °C for 1 s and 60 °C for 20 s. To compare mRNA levels among samples, mRNA for each gene of interest was normalized to the expression of a housekeeping gene, *HPRT*, using the standard curve method. Real-time PCR reactions were performed with the Universal TaqMan 2× Fast Universal PCR Master Mix (Applied Biosystems) in a 20 μl reaction volume.

2.5. Immunohistochemistry

The right ovarian sections were deparaffinized, treated with 90% methanol containing 3% H_2O_2 for 10 min at room tempera-