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Melatonin stimulates the secretion of progesterone along with the expression of cholesterol side-chain cleavage enzyme (P450scc) and steroidogenic acute regulatory protein (StAR) in corpus luteum of pregnant sows

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ABSTRACT

The direct effect of melatonin on porcine luteal function during the pregnancy remains unknown. The objective of the study was to analyse the molecular mechanism(s) by which melatonin directly affects progesterone (P4) production in the corpus luteum (CL) of pregnant sows. We evaluated the localization of melatonin membrane receptors (MT1 and MT2) in CL, and investigated the effect of melatonin on P4 secretion along with the expression of P4 synthesis intermediates in luteal cells. Immunohistochemistry analysis showed that MT1 and MT2 were predominantly localized in luteal cells in pregnant luteal tissues. The results of our in vitro experiments showed that melatonin from 5 to 625 pg/mL was able to significantly increase P4 release ($P < 0.05$) in a dose-dependent manner. And at the dose of 125 pg/mL treatment, the time-dependent effect on P4 secretion was observed. Furthermore, melatonin from 5 to 625 pg/mL up-regulated both P450scc and StAR expression ($P < 0.05$) in a dose-dependent manner, and the effect was also time-dependent. No difference of 3β hydroxysteroid dehydrogenase (3β-HSD) expression was observed between control and treatment groups. In addition, melatonin induced a dose- and time-dependent promotion on cell viability. Additionally, the stimulatory effects of melatonin were blocked by luzindole, a non-selective MT1 and MT2 receptor antagonist, or partially blocked by a selective MT2 ligand, 4-phenyl-2-propionamidotetralin (4P-PDOT). The data support the presence of MT1 and MT2 in porcine CL and a regulatory role for melatonin in luteal function through MT1 and MT2-mediated signal transduction pathways.

1. Introduction

CL is a temporary functioning organ and plays a crucial role in the regulation of the estrous cycle and in the maintenance of pregnancy. The luteal function is carried out largely by P4, which is the main steroid hormone synthesized by this gland [1]. It is generally thought that pituitary LH plays a central role in regulating P4 secretion. However, reports also provide evidence of an essential modulatory role in P4 production by many other hormones and factors, including melatonin [2–4]. Melatonin, a pineal hormone, regulates the dynamic physiological processes, including sleep, circadian rhythm, sexual maturation and reproduction [5,6], but its role in luteal function in pregnant sows remains unclear. Melatonin modulates physiological functions at least through two molecularly distinct melatonin receptors, the MT1 and MT2 [7]. Many studies showed melatonin affected reproductive function, in part, through activation of receptor sites within the hypothalamic-pituitary-gonadal axis [8,9]. It is generally believed that the reproductive actions of melatonin are mediated by way of regulating gonadotropin release after effects on hypothalamic monoamine and GnRH [8,10,11]. It is well established that melatonin can influencegonadotroph secretion of LH and FSH [12]. Recently, more attention directed to the role of melatonin may play in the ovary. The effects of melatonin on ovarian function vary with cell type, tissue structure and whether the species is a seasonal or a nonseasonal

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breeder. Melatonin has been reported to increase P4 production by human granulosa cells and luteal cells [13,14]. Study by Nakamura et al. showed that increased endogenous melatonin in preovulatory human follicles does not directly influence P4 production [15]. In equine luteal cells, P4 secretion stimulated by eCG or forskolin was inhibited by melatonin [16]. Together, these observations suggest that the direct effect of melatonin on ovary P4 production could be highly complex and species dependent. Melatonin membrane receptors MT1 and MT2, are expressed in several ovarian cell compartments indicating that melatonin functions as an important regulator within ovary [17,18]. These findings provide further evidence that melatonin may regulate ovarian function through binding to melatonin membrane receptors, MT1 and MT2. However, the direct role of melatonin in porcine luteal function has not been reported, especially in pregnant ovary. Recent interest concerning the role of melatonin in regulating cellular proliferation and apoptosis in a number of different cells [19–22]. Studies show that melatonin binding is clearly concentrated around the follicles in which granulosa cells are known to proliferate [23]. In the pregnant rats, exogenous melatonin promotes endometrial development and embryo implantation [24]. To date, however, the role of melatonin as a regulatory agent in CL is still limited and the degree to which melatonin acts on proliferation in luteal cells are yet unclear. In the present study, we aimed to examine the potential direct regulatory role of melatonin in luteal function of pregnant sows, we first confirmed the expression of melatonin membrane receptors in luteal tissues and cultured luteal cells. To further understand the physiological significance of melatonin in the pregnant porcine CL, we investigated the effects of melatonin on the cell viability of luteal cells, the secretion of P4 and the expression of P450scc, StAR and 3β-HSD involved in steroidogenesis in cultured luteal cells.

2. Materials and methods

2.1. Animals

All experimental procedures involving animals were approved by an institutional animal care and local ethical committee. Ovaries were obtained from healthy sows at day 30 to day 40 of gestation at local abattoir (latitude 34°N), and the methodology for confirming the stage of pregnancy was estimated by measurement of the crownrump length, as described previously [25,26]. The ovaries were brought to the laboratory in chilled PBS containing 100 U/mL penicillin and 100 mg/mL streptomycin. The luteal tissues were dissected aseptically from the ovaries without any follicular contamination and the connective tissue were removed out and processed for immunocytochemistry and cell culture.

2.2. Immunohistochemistry

The luteal tissues were dissected aseptically from the ovaries and were fixed in neutral-buffered formalin for 24 h, then dehydrated through a graded series of ethanol and embedded in paraffin. Adjacent 6-mm frontal sections were mounted separately on gelatine-coated slides. Tissue sections were deparaffinized in xylene, and rehydrated in a graded series of ethanol. Nonspecific antibody binding was blocked by preincubation with 20% normal donkey serum for 30 min at 20 °C. Sections were sequentially treated with a commercial rabbit anti-human MT1 (bs-0027R) and rabbit anti-human MT2 (bs-0963R) primary antibody (Beijing Biosynthesis Biotechnology Co., LTD, China), diluted 1:200 in PBS, overnight at 4 °C. Followed by incubation with a secondary antibody biotinylated donkey anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min. Immunoreactivity was visualized using the chromogen 3,3-diaminobenzidine tetrahydrochloride (DAB) (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 0.035% in Tris buffer saline for 1 min at room temperature. All sections were counterstained with hematoxylin for nuclear staining. Finally sections were dehydrated, mounted and covered with glass coverslips. The specificity of immunoreactivity was verified by absence of immunostaining when primary antibodies were omitted.

2.3. Culture of luteal cells

The luteal tissues were minced into about 1 mm^3 small pieces and washed with chilled PBS. Then the luteal pieces were dissociated at 37 °C in Medium 199 (M199) (Gibco, Grand Island, NY, USA) containing 0.2% (w/v) collagenase type 1 (Sigma, St. Louis, MO, USA) for 40 min, and dispersed cells were separated from undigested tissue by filtration through a 150 mm mesh stainless steel screens followed by washing thrice with serum-free M199 to remove nondissociated tissue fragments. The cells purification was performed as previously described [14,27]. The purified cells were resuspended in M199 contained 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA) and then seeded in six-well Poly-L-Lysine-coated dishes for incubation at 37 °C in an atmosphere of 5% CO_2.

2.4. Cell treatments

To detect the expression of MT1 and MT2 protein in primary luteal cells, the cells were cultured on sterile slides for 24 h. After this incubation, the cells were fixed in 4% paraformaldehyde, washed with ice-cold PBS, then treated with 0.1% Triton X-100 for 10 min. After incubated with 5% BSA for 1 h, the cells were incubated with the primary antibodies, namely, anti-MT1 and anti-MT2, respectively. Primary antibodies were detected by FITC-conjugated and PE-conjugated anti-rabbit IgG (Santa Cruz). To detect the expression of MT1 and MT2 mRNA in luteal cells, we seeded cells in 6-well plates at a density of about 1.5×10^5 cells / well, and incubated under 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h. The cells were then harvested for analyzing the expression of MT1 and MT2 mRNA. A further experiment was conducted to detect the effects of melatonin on the secretion of P4 and the expression of P450scc, StAR and 3β-HSD, we seeded cells in 6-well plates at a density of about 1.5×10^5 cells / well, and incubated under 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h, the medium was changed, and luteal cells were cultured with different doses of melatonin (0, 1, 5, 25, 125 and 625 pg/mL) dissolved in 0.2% dimethyl sulfoxide (DMSO) for 24 h. Melatonin was obtained from Sigma (St Louis, MO, USA). Then media was collected for analysis of P4 concentration, cells were harvested for analysis of changes of P450scc, StAR and 3β-HSD expression. To determine the time course of P4 production and P450scc, StAR and 3β-HSD expression in response to melatonin in our culture system, experiments were performed to analyze the P4 concentration in the cultured media and the expression of P450scc, StAR and 3β-HSD in the cultured luteal cells following 0, 8, 16, 24, 32 and 40 h of exposure to melatonin (125 pg/mL, according the previous dose-response study and the porcine physiological melatonin concentration) treated. In order to study the receptors dependent mechanism of melatonin action in P4 secretion, we used a non-selective melatonin MT1 and MT2 receptor antagonist, luzindole, or a selective MT2 ligand, 4P- PDOT (St Louis, MO, USA) in cultured luteal cells. After 24 h in culture, the medium was changed, melatonin (125 pg/mL) and luzindole (10^{-7} mg/mL) or 4P-PDOT (10^{-7} mg/mL) were added. After 24 h of incubation in treatment medium (according previous time-course study), the media was collected for analysis of P4 level, and the cultured cells were harvested to detect