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Baboon corpus luteum: the effect of melatonin on in vitro progesterone production*†

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Objective: To determine the effect of melatonin on baboon corpus luteum (CL) cell progesterone (P) production.

Design: Five baboon CL obtained during the midluteal phase by luteectomy were dissociated using collagenase, and incubations were performed (50,000 cells per plate) in quadruplicate for 3 hours at 37°C with melatonin (0.001 to 1.0 ng/mL) (basal) or with melatonin and 10 IU of human chorionic gonadotropin (hCG) (hCG-stimulated). Total P was measured by a specific radioimmunoassay.

Main Outcome Measures: Progesterone concentrations measured in the presence and absence of melatonin and hCG.

Results: Melatonin (0.01 to 1.0 ng/mL) inhibited basal P production in all the CL (41.8 ± 9.9 ng P without melatonin compared with 32.2 ± 2.0 ng P, 28.4 ± 2.1 ng with 0.01 and 1.0 ng/mL melatonin, respectively). Human chorionic gonadotropin-stimulated P production was significantly inhibited with as little as 0.01 ng of melatonin (150.8 ± 11.4 ng with 10 IU hCG versus 120.3 ± 6.4 ng with 10 IU hCG and 1.0 ng melatonin). The degree of inhibition in the hCG-stimulated cells was greater than in the nonstimulated cells. Melatonin at a concentration of 0.001 ng/mL did not affect P production in both stimulated and nonstimulated cells. Serotonin in similar concentrations had no effect on luteal cell P production.

Conclusion: These findings indicate that melatonin exerts a suppressive effect on baboon dispersed luteal cell P production and thus may play a role in luteal function.

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Key Words: Melatonin, corpus luteum, progesterone, luteal function

In addition to pituitary luteinizing hormone (LH) and follicle-stimulating hormone, a number of peptides have recently been implicated in playing a role in the control of the ovary. Melatonin, an indole derivative chemically identified as *N*-acetyl-5-methoxytryptamine, is a neurohormone with a molecular weight of 232. Secreted by the mammalian pineal gland, melatonin has been shown to influence reproductive function in many mammalian species (1). Administration of melatonin to female rats diminishes ovarian weight (2), blocks ovulation, and suppresses the vaginal estrus cycle (3). Extended darkness, which prolongs melatonin synthesis, suppresses gonadal function in several mammalian species, particularly the rat and hamster (4, 5). In the lower vertebrates, evidence suggests that melatonin may modulate the release of hormones from the anterior pituitary gland. Pinealectomy results

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in an increase in the mitotic rate and enzymatic activity in the rat pituitary gland, suggesting that the secretion of the pineal may exert an inhibitory effect on the functional status of the rat pituitary gland (6). However, in the primates there is so far no defined function of melatonin. Recently, melatonin has been reported to be involved in human reproductive processes such as puberty (7) and the menstrual cycle (8–11). As in the rat, it is generally believed that melatonin exerts an antigonadotropic effect mainly at the level of the brain and pituitary gland. Given during the follicular phase, melatonin amplifies pulsatile LH secretion in women by augmenting LH pulse amplitude (12). Exogenous tritiated melatonin was also found to be in higher concentration in the ovaries of rats and cats (13). Furthermore, melatonin receptors have been reported in not only hamster and rat ovaries but also in the human (14).

To define the role of melatonin on primate reproduction, we investigated the effect of melatonin on progesterone (P) production by baboon corpus luteum (CL) in vitro.

MATERIALS AND METHODS

Experimental Animals

Five CL were obtained from adult female baboons weighing between 14 and 17 kg and with well-defined menstrual cycles. The tissue was obtained by luteectomy performed at laparotomy. Each animal was anesthetized with an intramuscular injection of ketamine (10 to 12 mg/kg) for induction followed by endotracheal intubation with a mixture of nitrous oxide and oxygen (1:1 by volume) with 2% to 4% halothane for maintenance. Each CL was immediately weighed and placed in Ham's F-10 media (GIBCO, Grand Island, NY). The CL examined in this study were from the midluteal phase of the cycle, collected 8 to 9 days after maximal tumescence; the day of maximal turgescence was taken as the day of ovulation. Daily blood samples for the measurement of plasma P were also drawn at the time of maximum turgescence and throughout the luteal phase up to the time of luteectomy.

Materials

Melatonin (*N*-acetyl-5-methoxy-tryptamine) was obtained from ICN Biomedicals, Inc., (Costa Mesa, CA). Human chorionic gonadotropin (hCG), Profasi, was from Serono Laboratories, Inc. (Randolph, MA). All tissue culture supplies were obtained from Grand Island Biological Co. (Grand Island, NY). These included Ham's F-10 medium, penicillin, streptomycin, and fungizone. Tissue culture grade *N*-[2-Hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]) (Hepes), trypsin inhibitor (types 1 to 5 from soy bean), deoxyribonuclease (DNase) (Type 1 from bovine pancreas), bovine serum albumin (BSA), and serotonin (3-[2-Aminoethyl]-5-hydroxyindole) were purchased from Sigma Chemical Co. (St. Louis, MO). Collagenase (CLSI) was obtained from Worthington Diagnostic Systems, Inc. (Freehold, NJ). (1, 2, 6, 7-H^3) P with a specific activity of 91.8 curies/mmol was obtained from Amersham Corporation (Arlington Heights, IL).

Preparation of Dispersed Luteal Cells

The CL was immediately finely minced in Ham's F-10 medium. The cells were dispersed by incubating the minced tissue in Ham's F-10 medium at 37°C in a shaking water bath. The medium used for disaggregation of the luteal cells contained 10% BSA, 20 mM Hepes, 0.02% calcium lactate, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg fungizone, 100 μg DNase, 150 μg trypsin inhibitor, and 800 U collagenase/g of tissue. For each 100 mg of tissue, 1 mL of medium was employed. The tissue minces were flushed through sterile Pasteur pipettes to speed up the dispersion. Incubations were performed in air and at 10-minute intervals; the supernatant containing the disaggregated cells was removed. The cells were centrifuged and the pellets taken up in Ham's F-10 media. This procedure was repeated four to five times, and the final cell suspension was filtered through a nylon mesh (105 μm). The filtrate was centrifuged at 4°C for 10 minutes at 100 × *g*, and separate aliquots of the suspended cells were counted in a hemocytometer and treated with Trypan Blue for the determination of cell viability (15). The final volume of the suspension was adjusted with medium to give 100,000 cells/100 μL.

Incubation of Dispersed Cells With Melatonin and HCG

To determine the effect of melatonin, aliquots of the cell suspension (50 μL containing 50,000 cells) were placed into separate wells, in quadruplicate for each experimental point, in a sterile multiwell disposable plate. The doses of melatonin used ranged from 0.001 ng to 1.0 ng/mL, equivalent to 4.3 pmol to 4.3 nmol. The total volume per well was 1 mL. In parallel with melatonin alone, the cells were also incubated with 10 IU of hCG with the above doses

of melatonin to determine the effect of melatonin on hCG-stimulated P production by luteal cells. Previous experiments in our laboratory have established that optimal conditions required 50,000 to 100,000 cells per incubate and 1 to 10 IU hCG with 3 hours of incubation to obtain maximal P production (16). The cells were incubated for 3 hours in air at 37°C in a shaking water bath, and the reaction terminated by rapid freezing of the media and cells at −70°C. Viability of the cells determined both at the beginning and at the end of the incubation period showed that >80% of the dispersed luteal cells were viable and remained so at the end of the experiment. To determine the specificity of the inhibition, serotonin in similar concentration was also added to parallel incubations.

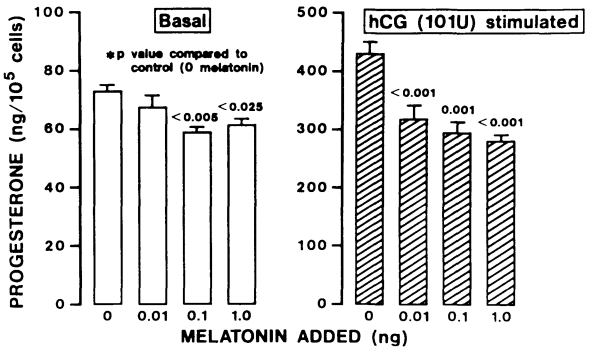

Figure 1 Effect of melatonin on basal and hCG-stimulated (10 IU) P production by dispersed baboon luteal cells from a midluteal phase CL. Each bar value represents the mean ± SEM of quadruplicate incubations (CL 1). Note the 2.5-fold difference in the scale of the P concentration between the basal and hCG-stimulated luteal cells. Melatonin (0.01 to 1.0 ng) = 43 pmol to 4.3 nmol.

Determination of P

Total P (cells and medium) was determined by a sensitive radioimmunoassay that has been validated and previously described (16). The antisera used was raised in rabbits against P-11-oxime BSA conjugate. Aliquots of the incubates were extracted with diethyl ether, and procedural losses from the extraction were monitored using tritiated P. The incubates were individually extracted, the extract was dried and resuspended in buffer solution for determination of P. All P values were corrected for recovery for extraction losses. Progesterone production by luteal cells was expressed as ng P per 100,000 cells. The P concentration in the cells at time 0 is consistently <2 ng/mL. This value was subtracted from the final data. Thus, the P concentration shown is P produced during the 3 hours of incubation.

Statistical Analysis

Data for total P were expressed as means ± SE. Means were compared by one-way ANOVA followed by the paired *t*-test. A P value ≤ 0.05, derived from a two-tailed probability value, was considered significant.

RESULTS

Figure 1 shows the effects of different concentrations of melatonin on in vitro P production by dispersed baboon luteal cells from a midluteal phase CL. In this CL the luteal cells in the absence of melatonin (control) produced 72 ng P per 100,000 cells in 3 hours. Addition of 0.001 ng (4.3 pmol) (not shown in Fig. 1) and 0.01 ng (43 pmol) of melatonin did not significantly affect P production when compared with the control. A significant inhibition of P production was obtained with addition of 0.1 ng melatonin (430 pmol). This inhibitory effect on P production was not further accentuated but remained similar when the dose of melatonin added was increased to 1.0 ng of melatonin (4.3 nmol). In contrast, 0.01 ng melatonin (43 pmol) decreased hCG-stimulated P output from 425 ng to 310 ng per 100,000 cells ($P < 0.001$). A further reduction in P production is seen with increasing doses of melatonin.

In a second midluteal phase baboon CL, the findings were essentially similar in pattern to those obtained in Figure 1; however, a considerable variation in the amount of P produced by CL of similar luteal age was observed even when standardized for in vitro P output by dispersed luteal cells under experimental conditions. The amount of P under basal conditions in the absence of melatonin was 42 ng per 100,000 cells. In the presence of 1.0 ng of melatonin, the amount of P produced was 30 ng/100,000 cells and was significantly different. In the presence of 10 IU of hCG, P production increased fivefold (150 ng/100,000 cells). A significant decrease was observed in the presence of 0.01 to 1.0 ng of melatonin (115 and 110 ng of P per 100,000 cells, respectively).

When the cumulative data from all five midluteal phase CL are examined, a significant inhibitory effect on P production is demonstrated at 0.01 to 0.1 ng melatonin on basal P production and on hCG-stimulated P production. To express the effect of melatonin on all the five CL as a whole and to obviate large differences in P production by different CL, the P data for each CL have been transformed

as a percentage of the corresponding basal or hCG-stimulated controls (without melatonin). Figure 2 shows the inhibitory effect of melatonin on in vitro P production by luteal cells from separate experiments on five different midluteal phase CL. Both basal and hCG-stimulated P production are significantly inhibited by 0.01 ng (43 pmol) melatonin (23%) to reach maximum inhibition (32%) with 0.1 ng (430 pmol). The degree of inhibition by melatonin is greater when the luteal cells are stimulated by hCG. At the higher dose of 1.0 ng (4.3 nmol) of melatonin, it is noteworthy that although both basal (16%) and hCG-stimulated P production (12%) are still significantly inhibited, there is partial loss of the suppressive effect when compared with 0.1 ng (430 pmol) of melatonin. In parallel experiments, serotonin did not show any effect on P production both in the hCG-stimulated and nonstimulated cells.

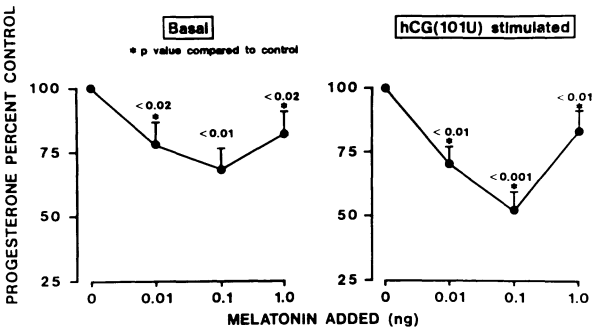

Figure 2 Melatonin inhibition of in vitro P production by luteal cells from baboon CL of the menstrual cycle. The P values represent the mean ± SEM obtained from separate experiments on five different midluteal phase CL. For each CL, quadruplicate incubations were performed for each dose of melatonin added. To obviate large differences in P output by different CL, all data on P production from each CL were individually transformed to a percentage of the corresponding controls (no melatonin) for the non-hCG (basal) and hCG-stimulated cells.

DISCUSSION

The present experiments demonstrate that melatonin, in concentrations similar to the plasma levels found during the human menstrual cycle, has a direct action on in vitro luteal cell P production of midluteal phase CL of the baboon. This P inhibitory effect of melatonin was observed with as little as 0.01 ng melatonin in non-hCG-stimulated cells and hCG-stimulated cells. These concentrations of melatonin are in the physiological range and are similar to human serum melatonin levels of 0.2 to 0.6 nmol during the follicular phase and 0.2 to 0.8 nmol during the luteal phase (10). That melatonin inhibits primate luteal cell P production is consistent with the antigonadal effect of melatonin found in subprimate species, generally believed to be mediated at the hypothalamo-pituitary level (2, 3, 6). Melatonin has also been shown to block hCG stimulation of rabbit ovarian follicle steroidogenesis (P, testosterone, and androstenedione) (17) and to inhibit rat testicular androgen synthesis (18).

Our findings disagree with the in vivo P stimulating effect of melatonin reported in early pregnancy CL of the marmoset monkey (19). The apparent difference may be due to the use of CL studied between 18 and 20 days after ovulation in four pregnant monkeys, the concentrations of melatonin perfused, which were estimated as high physiological levels, and the primate species employed in that study. A stimulatory effect of melatonin is also seen in vitro in human granulosa cells using higher doses of melatonin (200 pg/mL) (20). In the present study using the baboon CL, a stimulatory effect of melatonin on P production is also seen at a dose of 1 ng. It is quite evident that there are considerable differences in the P output between CL of the nonpregnant primate even when standardized for experimental conditions and luteal cell numbers from CL at similar phases of the menstrual cycle, as shown in the present study. Furthermore, primate CL at different stages of the luteal phase may exhibit different effects on P production by the same potential luteal modulator as previously reported for prostaglandins (21). Thus the CL of early pregnancy, which has been rescued by the trophic stimulus for early gestation, may respond differently.

Thus far, there is no evidence for ovarian production of melatonin but human preovulatory follicular fluid has higher concentrations of melatonin than peripheral blood, suggesting an intraovarian concentrating mechanism for melatonin (22). Therefore, pineal melatonin may be modulating luteal function through its action on the brain and pituitary as well as a direct effect on the ovary. Luteal phase human serum melatonin levels are higher than in the follicular phase, and there is good correlation between serum P and melatonin levels (10). In female runners in whom luteal function can be impaired, running increases melatonin levels (23, 24). The mechanism and site of melatonin action on luteal cell steroidogenesis are not known. There is suggestive evidence for the presence of cytoplasmic melatonin receptors in human ovaries (14) through which it may affect steroidogenesis, or, alternatively, it may have a direct action on the steroidogenic pathway. Because there is a greater inhibitory effect

of melatonin on hCG-stimulated luteal cell P production, melatonin may exert its effect through a similar but antagonistic mechanism of action as hCG on P production.

In conclusion, we have shown a significant direct inhibitory effect of melatonin on both basal (unstimulated) and hCG-stimulated luteal cell P output in vitro from the midluteal phase baboon CL. Thus melatonin may be a putative modulator of luteal function.

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