Melatonin directly stimulates the secretion of progesterone by human and bovine granulosa cells *in vitro*

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Summary. Melatonin, at concentrations and periods of exposure reflecting those present during the circadian cycle, was investigated for its influence on steroid production by granulosa cells cultured in serum-supplemented medium. At high (200 pg/ml) but not low (20 pg/ml) physiological concentrations, melatonin significantly stimulated progesterone production by human granulosa cells. This response was independent of the overall level of cell activity and was seen under the different culture conditions associated with different culture media. Exposure to melatonin for 8 h significantly stimulated progesterone secretion to a level similar to that achieved under continuous exposure, and the effect was reduced to control levels during subsequent periods in which no melatonin was added. Melatonin had no consistent effect on aromatase activity in the conversion of stored or serum-available androgen to oestradiol.

Melatonin significantly stimulated progesterone production by bovine granulosa cells *in vitro*, at concentrations similar to those present during the endogenous nocturnal rise (100–400 pg/ml). This response to physiological conditions by human and bovine cells suggests a role for melatonin in the regulation of progesterone production by the ovary.

Introduction

The influence of the pineal gland on reproductive function has been shown to be mediated by its major hormonal product, melatonin (Cardinali, 1981). The evidence available concerning the site and mechanism of melatonin action is conflicting but suggests that the overall response may be the result of action at central and peripheral sites in the hypothalamic–pituitary–gonadal axis. Early experiments described the distribution of tritiated melatonin; it becomes concentrated in the hypothalamus and mid-brain when administered into the CSF (Cardinali *et al.*, 1973) and enters all tissues, but concentrates only in the ovary, eye and pineal gland when injected systemically (Wurtman *et al.*, 1964). Putative receptors for melatonin have been described in membrane (bovine) and cytosol (rat) fractions of various brain regions (Cardinali *et al.*, 1979; Niles *et al.*, 1979) and in the cytosol fraction of hamster and rat gonadal tissue and human ovaries (Cohen *et al.*, 1978).

A direct gonadal response to melatonin has been reported previously, including stimulation of progesterone secretion from human corpus luteum slices treated with pharmacological doses (MacPhee *et al.*, 1975). Melatonin stimulated basal progesterone but not oestradiol secretion in short-term incubation of rat granulosa cells (Fiske *et al.*, 1984) but was inhibitory to testicular androgen production (Peat & Kinson, 1971; Ellis, 1972; Kano & Miyachi, 1976). No response to melatonin was seen in bovine luteal cells in short-term incubation (Battista & Condon, 1986). The physiological relevance of responses observed using pharmacological doses and short periods of exposure is questionable. We have therefore investigated the *in-vitro* action of melatonin on granulosa cells at concentrations and for periods of exposure which are likely to reflect those occurring during the circadian cycle.

Materials and Methods

Cell preparation

Human. Granulosa cells were taken from preovulatory follicles of women undergoing ovum transfer in the course of an in-vitro fertilization programme. Follicular maturation had been achieved using either clomiphene (Pergotime, Serono, Freiburg, F.R.G.) treatment from Days 3 to 7 of the cycle followed by hMG treatment from Days 8 to 11 or clomiphene from Days 2 to 5 of the cycle and a combination of clomiphene and hMG from Days 5 to 9. On Day 11 of the cycle the patients received 5000 i.u. hCG and the follicles were aspirated by laparoscopy 36 h later.

Follicular development was monitored by serum oestradiol concentrations and ultrasonography. Granulosa cells were recovered from follicular fluid and Hams F10 lavages of the follicle, after centrifugation at 250 *g* for 10 min. The cells from a single patient were combined in 20–30 ml culture medium (see details given below) and subjected to Percoll (Pharmacia, Uppsala, Sweden) separation to remove red blood cells as follows. The whole cell suspension was divided between two siliconized glass round-bottom tubes, under sterile conditions, and underlayered with 60% Percoll (15 ml/tube). The tubes were centrifuged at 1000 *g* for 20 min (no brake) and the white cell layer, at the medium–Percoll interface, was carefully removed from each tube with a Pasteur pipette. The pooled cells were resuspended in 20 ml medium and centrifuged at 250 *g* for 10 min. The medium was removed and the cells washed twice more with 20 ml medium. Before the final centrifugation, the cell suspension was filtered through sterile nylon mesh to remove any clumped cells. A sample of the final preparation was sonicated and used to determine the cell concentration by DNA estimation.

Bovine. Ovaries from cows in the non-luteal phase (no active corpus luteum in either ovary) were obtained from the Hamburg slaughter-house and transported to the laboratory at ambient temperature. Large, follicular-phase, obviously dominant, preovulatory follicles were selected, avoiding those showing morphological signs of atresia (for example, opacity, turbidity, presence of red blood cells in follicular fluid). The selection criteria for bovine follicles used in this laboratory have previously been shown to result in 86% avoidance of atretic follicles (Jungclas & Luck, 1986). The follicular fluid was aspirated by syringe and the granulosa cell layer was gently agitated to release the cells into a small quantity of culture medium. The resulting cell suspension and follicular fluid were centrifuged (10 min, 150 *g*) and the cell pellets combined, before washing twice more in culture medium and sampling for estimation of cell concentration. This procedure resulted in $8\text{--}15 \times 10^6$ cells per follicle; further details concerning follicle selection and cell preparation have been reported previously (Jungclas & Luck, 1986).

Cell culture

The cells were cultured in multiwell plates (Nunc, Wiesbaden, F.R.G.) at a concentration of 2×10^5 cells/ml medium⁻¹.well⁻¹ in replicates of 6 wells per treatment. The culture media that were used during the study were RPMI 1640, Dulbecco's modification of Eagles medium (DMEM) or a 1:1 (v/v) mixture of DMEM and Hams F12 (all from Flow Laboratories, Meckenheim, F.R.G.) each containing 10% human male serum (human experiments) or fetal calf serum (Gibco, Karlsruhe, F.R.G.; cow experiments). Human male serum was obtained from blood collected at midday when melatonin concentrations are at a minimum. The serum was inactivated at 56°C for 30 min and sterilized by passage through a 0.2 µm membrane filter (Schleicher & Schuell, Dassel, F.R.G.). Melatonin (Sigma, Deisenhofen, F.R.G.) treatments were added in ethanolic solution, with an equivalent quantity of ethanol (1 µl/ml medium) being added to control wells. Cell attachment occurred within 2–4 h. Cultures were maintained for up to 4 days at 37°C in a humidified incubator (95% air/5% CO₂) with changes of media at 8 or 24 h as indicated for each experiment and the samples stored at –20°C to await assay.

Assays

Melatonin. The concentration of added melatonin in treatment media was determined by a direct RIA as previously described (Webley *et al.*, 1985). This assay has a sensitivity of 8.6 pmol and intra- and inter-assay coefficients of variation of <6.0% and <16% respectively. In all experiments, the expected concentrations of melatonin were found in the medium after culture with granulosa cells.

Steroids. The concentrations of progesterone and oestradiol were measured by direct RIA kits purchased from RSL Inc. (Carson, CA, U.S.A.) and Sorin Biomedical (Saluggia, Italy) respectively. These assays had working ranges and inter- and intra-assay coefficients of variation as follows: progesterone, 0.6–65 pmol/ml, 13%, 7.6%; oestradiol, 0.1–2.2 pmol/ml, 7.0%, 6.0%. The antibodies used in the kits showed no cross-reaction with melatonin.

DNA. Cell number was calculated from estimation of DNA concentration according to the relationship $1 \mu\text{g DNA} = 1.67 \times 10^5$ cells. The bisbenzimidazole fluorescence method of Labarca & Paigen (1980) was used, with calf thymus DNA (Sigma) as standard. Diluting the sample from a high concentration, using assay buffer, removed the significant background effect of culture medium.

Statistics

Steroid concentrations in spent media were normalized for the number of cells present at the start of culture. Progesterone secretion by human and bovine granulosa cells over 24 h has previously been shown to be linearly related to cell number over a range of at least 1.5×10^4 to 1×10^6 cells per ml incubation volume (M. R. Luck, unpublished data). The data are expressed as mean $\pm$ s.e.m. and Student's unpaired *t* test was used to determine statistical significance between control and treatment groups.

Results

The influence of a range of melatonin concentrations, from 0 to 800 pg/ml, on progesterone and oestradiol secretion by human granulosa cells cultured for 4 days in DMEM is shown in Table 1. There was no clear dose-response relationship between melatonin and progesterone but progesterone secretion was significantly stimulated by the two highest melatonin concentrations of 200 and 800 pg/ml whereas melatonin concentrations of <200 pg/ml had no consistent effect on progesterone secretion. There was no consistent effect, at any concentration of melatonin, on oestradiol production.

Melatonin concentrations of 20 and 200 pg/ml were chosen to represent low and high physiological doses. The effects of these two melatonin concentrations on progesterone and oestradiol production by human granulosa cells cultured in RPMI 1640 are shown in Fig. 1. These results may be compared to those from cells exposed to similar doses of melatonin in DMEM, shown in Table 1. There was a difference between the concentrations of progesterone secreted in the two culture media and in the behaviour of the cultures. In RPMI 1640, progesterone secretion in the absence of melatonin declined during the culture period whereas in DMEM progesterone production was greater and increased to a peak on Day 3 of culture. Under both culture conditions, a high physiological concentration of melatonin (200 pg/ml) significantly stimulated progesterone production, on Days 2 and 3 of culture in RPMI 1640 and Days 1 to 3 in DMEM. Melatonin at a low physiological dose had a small but insignificant stimulatory effect on progesterone secretion and there was no consistent influence, in either culture, on oestradiol production.

These experiments demonstrate the effect of melatonin during continuous exposure. To investigate the influence on progesterone secretion with a period of melatonin exposure similar to that likely to be occurring *in vivo* during the circadian cycle, media were changed at 8-h intervals. Cells

Table 1. Concentrations of progesterone and oestradiol in culture medium from human granulosa cells cultured in the presence of various concentrations of melatonin

		Melatonin (pg/ml)						
		Day 0 (control)	12.5	25	50	100	200	800
Progesterone (nmol.µg DNA ⁻¹ .ml ⁻¹ .24 h ⁻¹)	1	4.5 ± 0.3	5.1 ± 0.4	5.5 ± 0.7	4.9 ± 0.3	6.0 ± 0.3**	6.5 ± 0.2***	6.0 ± 0.4*
	2	6.1 ± 0.7	7.3 ± 0.4	7.3 ± 0.3	5.6 ± 0.2	6.5 ± 0.3	8.7 ± 0.2**	8.8 ± 0.5**
	3	8.7 ± 0.6	8.0 ± 0.4	8.9 ± 0.6	8.0 ± 0.5	8.9 ± 0.8	11.2 ± 0.4**	12.0 ± 0.4**
	4	7.7 ± 0.5	7.4 ± 0.6	8.2 ± 0.5	7.6 ± 0.2	8.3 ± 0.4	9.1 ± 0.5	9.2 ± 0.7
Oestradiol (pmol.µg DNA ⁻¹ .ml ⁻¹ .24 h ⁻¹)	1	51.5 ± 1.9	52.2 ± 2.2	47.0 ± 1.8	57.0 ± 3.3	54.8 ± 1.8	54.8 ± 1.8	54.4 ± 3.3
	2	17.6 ± 0.4	18.4 ± 0.7	19.9 ± 1.1	21.7 ± 0.7	18.4 ± 0.7	21.7 ± 0.7**	20.2 ± 0.7**
	3	16.5 ± 0.4	14.7 ± 1.1	14.3 ± 0.4**	15.8 ± 0.7	16.9 ± 0.3	16.2 ± 0.3	16.9 ± 0.7
	4	16.2 ± 0.7	15.1 ± 0.2	15.1 ± 0.2	17.6 ± 0.7	15.4 ± 0.7	14.7 ± 0.3	15.4 ± 0.4

Values are mean ± s.e.m. for 6 observations.

P* < 0.05, *P* < 0.01, ****P* < 0.001 compared with control values.

Fig. 1. Concentrations of progesterone and oestradiol produced by human granulosa cells cultured in medium RPM 1640 in the presence of low (20 pg/ml) and high (200 pg/ml) physiological concentrations of melatonin. Values are mean ± s.e.m., *n* = 6 replicate wells per treatment. Significance of values compared with control values: **P* < 0.05, ***P* < 0.01.

Fig. 2. Progesterone concentrations in culture media from human granulosa cells with changes of media at 8-h intervals. Treatments are: —control, ethanol (E), —melatonin (M, 200 pg/ml), —melatonin during the second 8-h period only. Significance of values compared with control (mean $\pm$ s.e.m.): * $P < 0.05$, $n = 6$ replicate wells per treatment.

Fig. 3. Progesterone concentrations in culture media from bovine granulosa cells cultured in various concentrations of melatonin. Values are mean $\pm$ s.e.m., $n = 6$ replicate wells per treatment. Significance of values compared with control values: * $P < 0.05$, ** $P < 0.01$.

were exposed either to a continuous melatonin concentration of 200 pg/ml or to an 8-h period of exposure to 200 pg melatonin/ml followed by 2 periods with no added melatonin. These cells, cultured in DMEM, produced lower progesterone concentrations than those measured in the previous culture, an overall decline in output probably reflecting the frequent changes in medium. However, continuous exposure to melatonin again significantly stimulated progesterone secretion, a small increase occurring after 8 h which reached significance after 16 h of culture (Fig. 2). Exposure to melatonin for an 8-h period stimulated progesterone secretion to a level similar to that achieved under continuous exposure, and the effect was removed during the subsequent periods in which no melatonin was added.

Bovine granulosa cells were cultured in DMEM in a similar manner to human granulosa cells. There was a significant increase in progesterone in response to melatonin at doses from 100 to 400 pg/ml with the maximum response at 200 pg/ml (Fig. 3).

Discussion

This study has described the effects of melatonin, at the physiological concentrations found *in vivo* during the circadian cycle (Webley *et al.*, 1985), on steroid production by human granulosa cells which had been induced to secrete luteal quantities of progesterone by being cultured in serum-supplemented medium. Melatonin, at high but not low physiological concentrations, significantly stimulated progesterone secretion. This response was independent of the overall level of cell activity and was seen under the different culture conditions (for example, differing energy content and amino acid composition) associated with different culture media. Although a linear dose-response relationship between melatonin concentration and progesterone secretion was not seen, the results suggest that the stimulation of progesterone secretion is dose-dependent and may start to occur at concentrations likely to be present during the dark phase of the circadian cycle (between 100 and 200 pg/ml). The stimulation of progesterone secretion became apparent after 16 h of culture (see Fig. 2); it occurred with continuous or limited (8 h) exposure to melatonin and could be reversed by melatonin removal. Taking an exposure period of 8 h to represent that experienced during the night-time elevation of the circadian cycle, this cycle of treatment suggests that stimulation of progesterone secretion can occur under physiological conditions in terms of both the concentration of melatonin and the period of exposure.

The cow shows a nocturnal rise in melatonin secretion of similar magnitude to that seen in humans (Martin *et al.*, 1983). Our observation of a stimulation of progesterone secretion by bovine granulosa cells treated with less than 1 nmol melatonin therefore shows that the effectiveness of physiological concentrations is not specific to human cells. The lack of response of bovine mid-luteal cells to higher concentrations of melatonin (10^{-5} to 10^{-7} M), reported by Battista & Condon (1986), may reflect the short period of exposure used. Alternatively, responsiveness may be confined to granulosa or granulosa-derived cells and is therefore lost or reduced by the mid-luteal phase (Schwall & Niswender, 1985).

Our observation of a stimulation of progesterone secretion but not of oestradiol secretion in human cells is consistent with that observed in the rat (Fiske *et al.*, 1984). The lack of aromatase stimulation was not the result of substrate limitation since male serum was used to supplement the cultures and would have provided androgen. A granulosa cell stimulation confined to the progesterone compartment therefore suggests a number of possible mechanisms for melatonin action, including either a change in the rate of uptake of lipoprotein-bound cholesterol from serum, a change in the rate of intracellular lipoprotein catabolism and conversion of cholesterol to progesterone, or a change in the rate of de-novo synthesis of progesterone from acetate. MacPhee *et al.* (1975) described the stimulation of progesterone production by human corpus luteum slices, with pharmacological doses of melatonin and observed an increase in the incorporation of labelled acetate into progesterone. It therefore appears that melatonin may be able to influence de-novo

synthesis but does not exclude the possibility that it also has a direct effect on cholesterol metabolism. It is also possible that the responsiveness of granulosa cells to melatonin may be potentiated by gonadotrophins, as reported for the rat by Fiske *et al.* (1984). In a previous investigation of the circadian pattern of melatonin during the human menstrual cycle (Webley & Leidenberger, 1986), a significant increase in the circadian melatonin output was observed during the luteal phase of the cycle. Higher melatonin concentrations were also recorded in subjects taking exogenous progestagens in the form of the contraceptive pill. These studies, together with the present results, indicate a positive relationship between melatonin and progesterone. Melatonin, via its direct action on the ovary, may increase the output of progesterone which in turn feeds back on the pineal gland to increase melatonin secretion. A change in the circadian pattern of progesterone secretion has not been reported in man but a nocturnal increase in secretion has been described for the rhesus monkey (Spies *et al.*, 1974; Healy *et al.*, 1984). A mutual reinforcement between the two hormones may provide a mechanism through which the pineal gland could influence menstrual cyclicity.

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