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Direct influence of melatonin on steroid, nonapeptide hormones, and cyclic nucleotide secretion by granulosa cells isolated from porcine ovaries

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Abstract: The release of progesterone, estradiol-17β, oxytocin, arginine-vasopressin, cAMP, and cGMP by cultured granulosa cells isolated from porcine ovaries without and in the presence of melatonin (0.001, 0.01, 0.1, 1, 10, and 100 ng/ml medium) was analyzed. It was found that melatonin is able to inhibit progesterone and stimulate estradiol secretion. Melatonin treatments significantly inhibited oxytocin release. Some inhibition of vasopressin and cAMP and significant stimulation of cGMP also resulted from melatonin treatment. The present observations suggest a direct effect of melatonin on the steroid, nonapeptide hormone, and cyclic nucleotide release from porcine ovarian cells.

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Introduction

The involvement of melatonin in the control of mammalian [Reiter, 1980; Arendt, 1986] and avian [Gwinner et al., 1981] reproductive cyclicity is well-known. It was demonstrated that the synthesis of this hormone from another indoleamine, serotonin, during the hours of darkness may play an important role in the photoperiodic regulation of reproduction in temperate mammalian species. In particular, there is evidence that melatonin can regulate gonadotropin output from the pituitary through the influence on hypothalamic monoamine and LH-RH production [Pavel, 1978; Reiter, 1980; Arendt, 1986]. It was demonstrated that melatonin and serotonin treatments influence not only the hypothalamo-hypophysial system, but also change the LH receptor content in rat ovaries [Trentini et al., 1976]. This effect may be due to indoleamine action at the hypothalamo-hypophysial or at the peripheral level.

The presence of melatonin in plasma [Kennaway et al., 1977] and ovarian follicular fluid [Brzezinski et al., 1987] can indirectly suggest a direct indoleamine influence on the ovaries. This supposition was confirmed by the observation that melatonin treatment prevented the fall of progesterone concentration in the monkey plasma after blockade of gonadotropin output by an LH-RH antagonist [Webley et al., 1991].

In vitro experiments demonstrated a direct effect of melatonin on the gonads. In particular, its addition to culture medium inhibited testosterone secretion by mouse [Kano and Migashi, 1976; Persengijev and Kehajova, 1991] and hamster [Niedziela and Lukaszyk, 1993] Leydig cells. Melatonin stimulated progesterone secretion by rat [Fiske et al., 1984], bovine [Webley and Luck, 1986], human [Webley and Luck, 1986; Brzezinski et al., 1991, 1992], and ovine [Baratta and Tamanini, 1992] granulosa cells in culture. No melatonin influence on estradiol release was observed in human, bovine [Webley and Luck, 1986], and ovine [Baratti and Tamanini, 1982] cell experiments. Ovine luteal cells, in contrast to granulosa cells, did not respond to melatonin treatment [Baratti and Tamanini, 1992]. Thus, a stimulatory effect of melatonin on progesterone, but not estradiol, release by cultured granulosa cells of these species was demonstrated. No reports concerning the effects of indoleamine on other important ova-

rian substances (peptide hormones, cyclic nucleotides) are available.

The objective of our in vitro experiments was to investigate the direct effects of melatonin on steroid (progesterone, estradiol-17β), nonapeptide hormones (oxytocin, arginine-vasopressin), and cyclic nucleotide (cAMP, cGMP) secretion by porcine granulosa cells.

Material and methods

The ovaries of Slovakian white gilts, 8 months of age, were collected after killing at a local slaughterhouse. Granulosa cells were collected by syringe aspiration from 2–5 mm follicles in ovaries of the early and middle follicular phases of the estrous cycle. After the separation of follicular fluid by centrifugation at $200 \times g$ for 5 min, the cells were washed three times in sterile TCM-199 supplemented by 10% bovine fetal serum (Veterinary Institute, Brno, Czechia), 10 mIU/ml insulin (Léčivá, Prague, Czechia), and 50 ng/ml gentamicin (Pharmacia, Sofia, Bulgaria). The cell suspension (1×10^6 cells/ml) was cultured 2 days in Linbro Plate wells (Flow Laboratories, Herts, UK), at 38.5°C in 5% CO_2 in humidified air. Thereafter the medium was replaced. The new medium had the same composition, but to which had been added 0.01, 0.1, 1, 10, 100, or 1,000 ng/ml of melatonin (Sigma, St. Louis, USA) and 10 ng/ml of 3-isobutyl-methyl-xanthine (inhibitor of cyclic nucleotide metabolism, Serva, Heidelberg, FRG). The original melatonin preparation was first dissolved in 0.2 ml of absolute alcohol and then in the incubation medium immediately before the experiment. The final content of alcohol in the medium did not exceed 0.001%. For the determination of cAMP and cGMP production the cells were cultured in the new medium for 12 hr (preliminary experiments having shown maximal amounts of this substances in the medium) at this time, then exposed to 10 min heating at 60°C to obtain maximal cyclic nucleotide output from the cells. The medium was aspirated and frozen to −40°C until cyclic nucleotide analysis. For the evaluation of hormone secretion, the incubation medium was aspirated after 2 days of the cell culture without or with melatonin treatment. Staining of the cells by Trypan blue at this period demonstrated 65–70% viability and the measurements of cell number showed no significant differences between the control and various experimental groups.

Hormone radioimmunoassays

Cyclic nucleotide, nonapeptide, and steroid hormone concentrations were determined in duplicate by RIA in each sample of medium conditioned by granulosa cells. The commercial kits from the Institute of Radioecology and Application of Nuclear Technics (URVIT, Košice, Slovakia) were used for progesterone and estradiol analysis. Oxytocin, vasopressin, cAMP, and cGMP were measured using RIA kits from Institute for Research, Production and Application of Radioisotopes (UVVVR, Prague, Czechia) following the instructions of the manufacturer.

Progesterone. The sensitivity of RIA was 2 pM/ml. Antiserum to progesterone crossreacted 58.6% with 11β-hydroxyprogesterone, 0.20% with corticosterone, less than 0.01% with cortisol, testosterone, estradiol-17β, estrone, and 4-androstene-3,17-dione. Intraassay coefficient of variation did not exceed 9%.

Estradiol. Sensitivity in this assay was 2.5 pM/ml. Crossreaction of antiserum used with estrone was 25%; with estriol, 1.84%; with 20-α-OH progesterone, 4-androstene-3,17–dione, testosterone, cortisol, cortisone, less than 0.001%. Intraassay coefficient of variation did not exceed 4%. The sensitivity of the radioimmunoassay for oxytocin was 1.2 pM/ml. The antiserum to oxytocin crossreacted less than 0.005% with arginine-vasopressin, 0.04% with lysine-vasopressin, 17% with arginine-vasotocin, and 22.6% with desamine-oxytocin. The intraassay coefficient of variation was between 5 and 9%.

Vasopressin. Sensitivity of determination was 1.2 pM/ml. Crossreaction of antiserum with lysine-8-vasopressin was 67.6%; with oxytocin, 0.025%; with vasotocin, 0.0025%. Interassay coefficient of variation varied between 4 and 7.0%.

cAMP. The sensitivity RIA was 0.05 pM/ml. Cross-reactivity of antiserum used with cGMP was less than 0.01%; with AMP, ADP, and ATP less than 0.003% of that to cAMP. Interassay coefficient of variation did not exceed 9%.

cGMP. Sensitivity was 0.05 pM/ml. The cross-reactivity of the antiserum used with cAMP, GMP, GDP, DTP, and ATP was less than 0.001%. Interassay coefficient of variation did not exceed 4%. None of the antisera was used for RIA cross-reactivity studies with melatonin.

For the measurement of I^{125} radioactivity, a gamma counter (LB MAG 312, Berthold, Germany) was used.

Statistics

The data presented are the values obtained in three separate experiments. In each experiment, a pool of

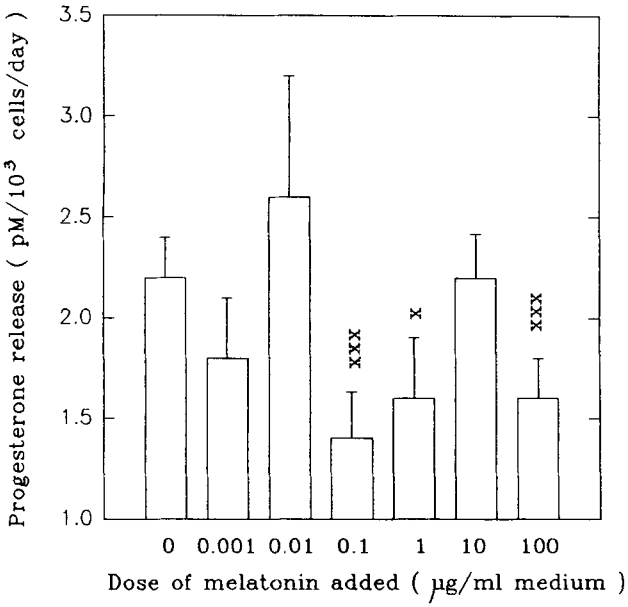

Fig. 1. Effect of melatonin on progesterone release by porcine granulosa cells in vitro. Values are means ± S.E.M., x—$P < 0.05$; xxx—$P < 0.001$ compared with control. Each dose of melatonin was tested on 12 cell cultures.

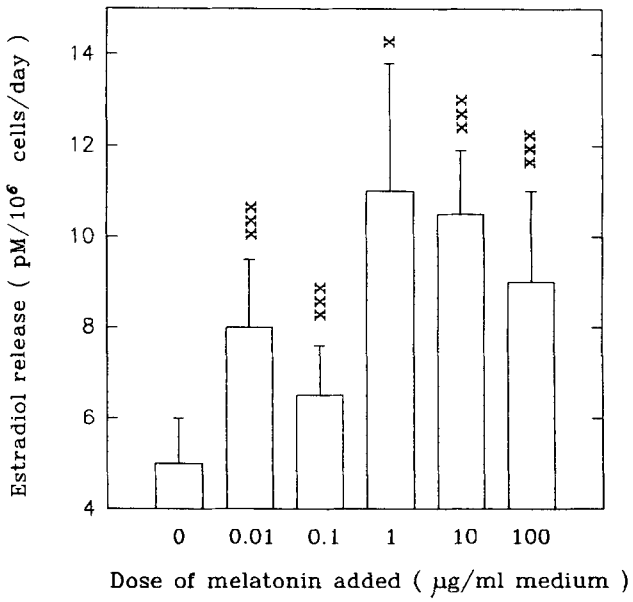

Fig. 2. Effect of melatonin on estradiol-17β release by porcine granulosa cells in vitro. Legends as in Figure 1.

granulosa cells isolated from 30 to 40 ovaries was used. Each experimental group was represented by four wells of granulosa cells. Rates of hormone and cyclic nucleotide secretion were calculated per 10^6 viable cells/day. Significant differences between the groups were evaluated with ANOVA test followed by multiple t-test.

Results

The effects of melatonin on steroid, nonapeptide hormones, and cyclic nucleotide release by granulosa cell cultures were examined in these experiments. High doses of the indoleamine (0.1, 1, and 100 ng/ml) decreased progesterone secretion significantly (Fig. 1). In contrast to progesterone, estradiol output was significantly increased after the addition of melatonin at concentrations 0.01, 0.1, 1, 10, or 100 ng/ml (Fig. 2). Melatonin decreased oxytocin secretion by the cells (Fig. 3). No clear influence of melatonin treatments on vasopressin output was observed with only one dose (0.1 ng/ml) causing a significant decrease (Fig. 4). Three doses of melatonin used (0.01, 10, and 100 ng/ml) caused a significant reduction of cAMP output. Melatonin given at other doses did not affect cAMP release (Fig. 5). All melatonin doses stimulated cGMP release by the cells with a statistically significant and dose-dependent effect over the range 0.1 to 100 ng melatonin/ml (Fig. 6).

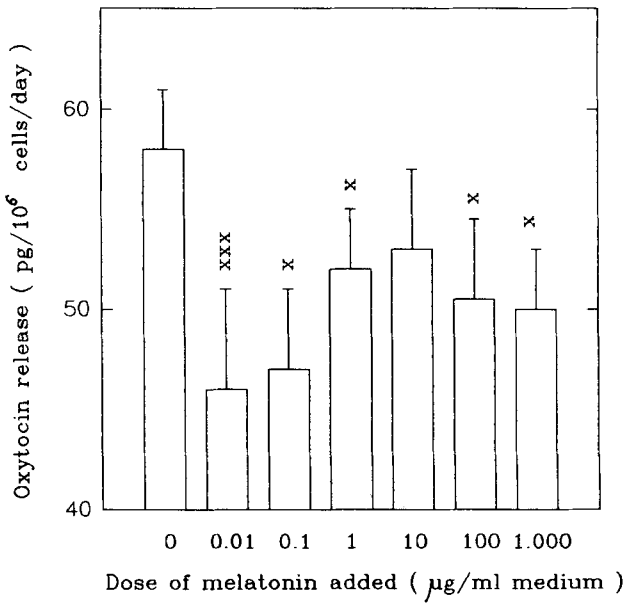

Fig. 3. Effect of melatonin on oxytocin release by porcine granulosa cells in vitro. Legends as in Figure 1.

Discussion

The results obtained here demonstrate a direct influence of melatonin on steroid, nonapeptide hormone and cyclic nucleotide release by porcine granulosa cells. Most melatonin doses decreased progesterone, increased estradiol, inhibited oxytocin, and have no significant effect on vasopressin release. Melatonin tended to decrease cAMP and stimulate cGMP output.

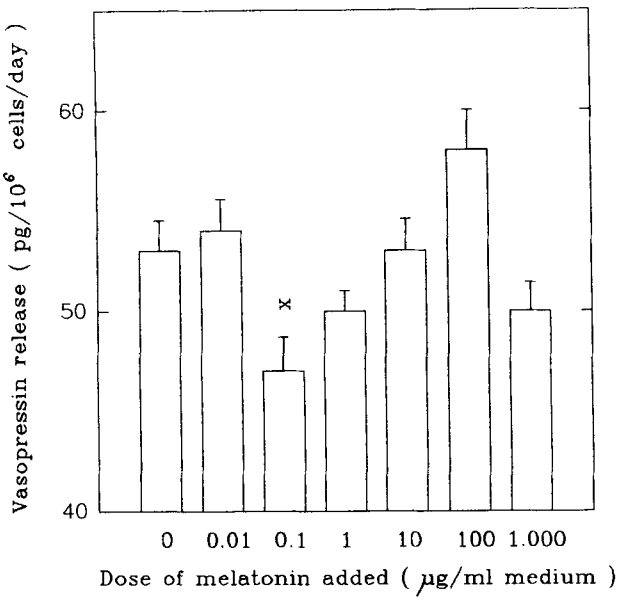

Fig. 4. Effect of melatonin on arginine-8-vasopressin release by porcine granulosa cells in vitro. Legends as in Figure 1.

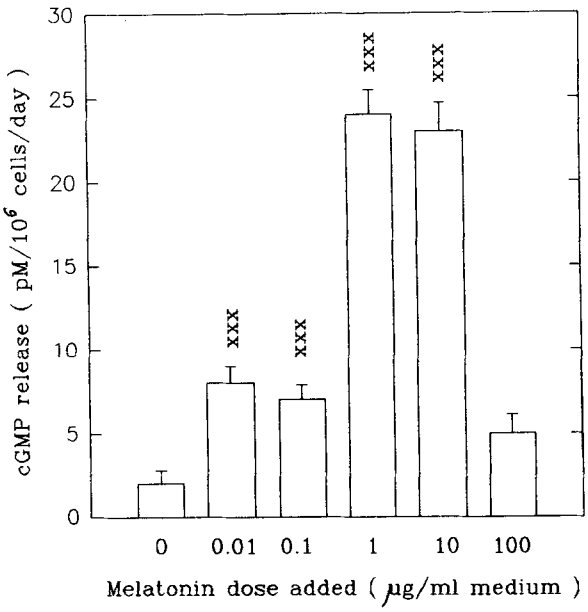

Fig. 6. Effect of melatonin on cGMP release by porcine granulosa cells in vitro. Legends as in Figure 2.

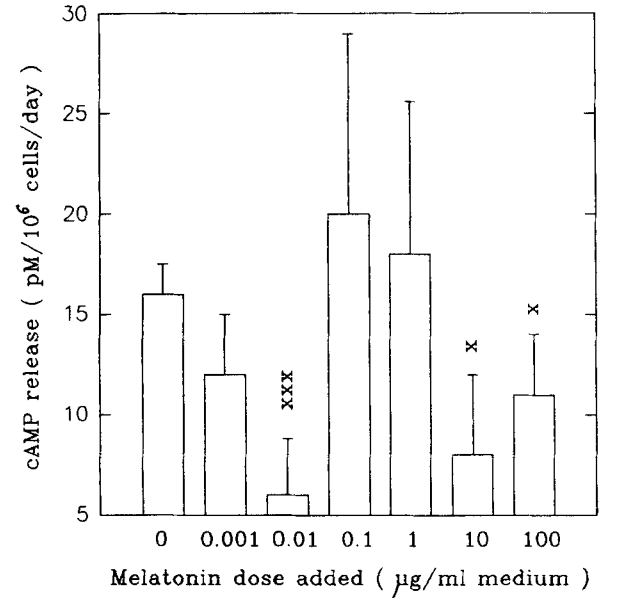

Fig. 5. Effect of melatonin on cAMP release by porcine granulosa cells in vitro. Legends as in Figure 2.

Since the low melatonin doses used in our experiments are comparable with the melatonin levels in mammalian blood, especially during its nocturnal rise [Kennaway et al., 1977; Reiter, 1980; Arendt, 1986] and in the follicular fluid [Brzezinski et al., 1987], our in vitro experiments may mimic processes that take place in vivo. Furthermore, the in vitro experiments can uncover direct effects of melatonin on the ovary usually masked by its central effects under in vivo conditions.

Our data on porcine granulosa cells concerning progesterone inhibition under the influence of melatonin differs from observations of progesterone-stimulatory effect in other species [Fiske et al., 1989; Webley and Luck, 1986; Brzezinski et al., 1991, 1992; Baratta and Tamanini, 1992]. On the other hand, in our experiments melatonin, in contrast to human, bovine [Webley and Luck, 1986], and ovine [Baratti and Tamanini, 1992] granulosa cells, actively stimulated estradiol release. These data suggest an alternate site of melatonin action on porcine ovarian steroidogenesis. In other species the indoleamine appears to stimulate the early stages of the steroidogenic pathway (progestagen synthesis), while in the pig later events (estrogen secretion) are affected. The intensive estrogen formation after melatonin addition may cause the depletion of precursors, in particular for progesterone, observed in our experiments. Normal ovarian follicles are characterized by an intensive synthesis of estrogens and while luteinized follicles and corpus luta produce mainly progestagens [Gore-Langton and Armstrong, 1988; Paton and Collins, 1992]. It may be supposed, that the physiological role of melatonin in pigs is the stimulation of folliculogenesis, while in other species investigated it may support the luteinization of follicular cells and corpus luteal development. Further investigations of steroidogenesis would be required to confirm these hypothesis.

Our observations appear to be the first evidence of a melatonin influence on ovarian nonapeptide hormone and cyclic nucleotide release. They are consistent with reports of melatonin inhibition of

hypothalamic oxytocin [Jasin et al., 1993] and hypophysial cAMP-dependent protein kinase [Hazlerigg et al., 1991]. The effects of melatonin on cyclic nucleotide release suggests the existence of a new mechanism of the action of lipid-soluble hormones since these hormones affect DNA directly through nuclear receptor [Richards and Hedin, 1988; Leung and Steele, 1992]. On the other hand, not only melatonin, but also other lipid-soluble hormones, steroid, can affect cAMP and cGMP release [Sirotkin and Nitray, 1993]. The effects of melatonin on nonapeptide hormone and cyclic nucleotide release may be involved in the regulation of steroid secretion since ovarian oxytocin [Wathes, 1989; Wathes and Denning-Kendall, 1992], cAMP and cGMP [Richards and Hedin, 1988; Leung and Steele, 1992] play important roles in the regulation of gonadal steroidogenesis and stimulation by gonadotropin. For example, the melatonin-induced inhibition of the secretion of oxytocin, an important stimulator of progestagen secretion [Sirotkin and Nitray, 1992 a,b; Wathes, 1989; Wathes and Denning-Kendall, 1992] may reduce progesterone release. The decrease of ovarian oxytocin secretion in turn may be induced by the reduction of cAMP output under the influence of melatonin since cAMP is known as a stimulator of ovarian oxytocin release [Wathes, 1989]. The observed melatonin influence on steroid release may be also mediated by cAMP- and/or cGMP-dependent intracellular mechanisms controlling steroidogenesis [Richards and Hedin, 1988; Leung and Steele, 1992] without the involvement of oxytocin.

In conclusion, the present observations demonstrate a direct influence of melatonin on the secretion of various ovarian regulators including steroids, nonapeptide hormones and cyclic nucleotides, although the interrelationships between the effects described remained to be investigated.

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