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Serotonin May Alter the Pattern of Gonadotropin-Induced Progesterone Release of Human Granulosa Cells in Superfusion System

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Serotonin plays a hormonal function in several nonneuronal peripheral tissues, such as the ovaries. Our aim was to investigate whether there is a modulatory action of serotonin on gonadotropin-induced steroid secretion of human granulosa cells. In granulosa cell culture, serotonin was administered alone or in combination with luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Also, granulosa cells were transferred into a dynamic superfusion apparatus and challenged by FSH and LH alone or along with serotonin. Estradiol and progesterone concentrations of samples were measured by radioimmunoassay. As expected, administration of FSH, LH, and serotonin alone resulted in a significant estradiol and progesterone release in cell culture, as well as a significant increase in progesterone release in dynamic superfusion system. In cell culture, co-administration of serotonin with gonadotropins had no additive effect on gonadotropin-induced secretion of progesterone, while it further augmented that of estradiol. In superfusion system, when gonadotropins were added along with serotonin, the increase in progesterone release was markedly less, while peaks of hormone response were remarkably prolonged compared to challenges by LH and FSH alone. The observed effects of serotonin on gonadotropin-induced steroid release of granulosa cells may reveal further details about the regulation of granulosa cell function.

Key Words: Serotonin; LH; FSH; granulosa cell superfusion; cell culture; estradiol; progesterone.

Introduction

There are experimental data demonstrating the presence and actions of various neurotransmitters in the ovary, thus supporting the view that they might play a role in intraovarian regulatory mechanisms. Serotonin (5-HT), in addition to its well-known effects within the nervous system, also plays a hormonal function in several nonneuronal peripheral tissues, such as the ovaries.

In the ovary of the teleost fish Medaka, 5-HT appears to have a regulatory effect on both steroidogenesis and oocyte maturation *(1)*, while in isolated bovine luteal cells, 5-HT stimulates progesterone production *(2,3)*. Furthermore, 5-HT was found to stimulate a dose-related increase in progesterone (P4) secretion of human granulosa cells (GCs) cultured in serum-free medium, with a maximal stimulatory effect at $10^{-4}M$ *(4)*. 5-HT has also been found in human follicular fluid (FF) *(5)*. In the ovary, 5-HT seems to stimulate progesterone production via 5-HT7 receptor, positively coupled to adenylate cyclase activity *(6)*.

Although much has been learned about the possible involvement of 5-HT in the regulation of ovarian steroidogenesis, no data are available to evaluate whether or not there is a potential modulatory action of 5-HT on gonadotropin-induced steroid secretion of human granulosa cells. To explore this, we examined human GCs in cell culture, as well as in a superfused cell system first introduced by Lowry for studying the hypothalamic–pituitary–adrenocortical axis *(7)*. The latter method has widely been applied to investigate the release pattern of various pituitary hormones under dynamic circumstances along with interaction between releasing factors and several brain peptides in a pituitary cell–superfusion system *(8)*. In the superfusion apparatus, cell function is investigated under dynamic in vitro circumstances, where hormones, metabolic, and waste products of cells cannot accumulate in the immediate milieu of cells, so non-physiological effect of substances in culture medium might be greatly reduced. Moreover, this experimental system, in combination with radioimmunoassay, has been proven to be a sensitive method to detect and demonstrate delicate hormone responses from GCs in response to low concentrations of different neurotransmitters *(9)*. Here we report our findings regarding the dynamic changes in the pattern of gonadotropin-stimulated P4 release from human GCs in response to 5-HT under in vitro circumstances.

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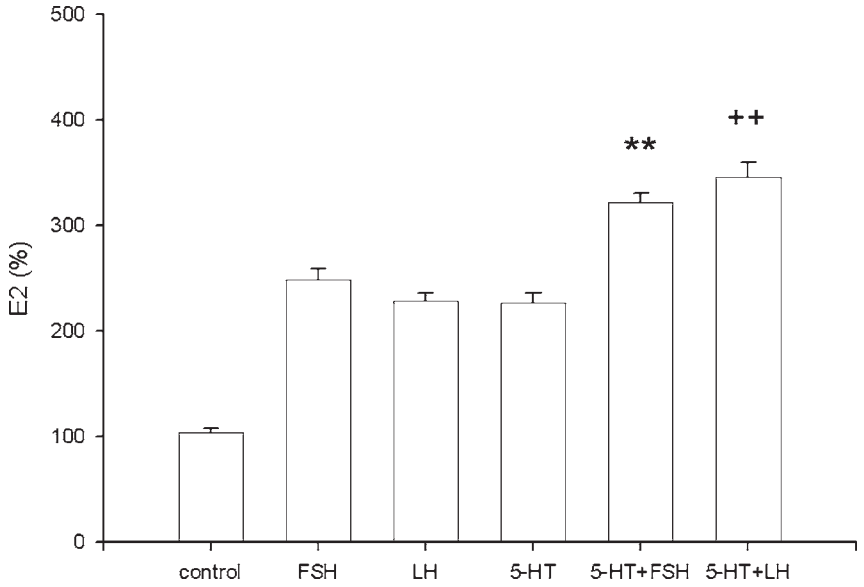

Fig. 1. Estradiol (E2) secretion in human granulosa cell culture treated with follicle stimulating hormone (FSH), luteinizing hormone (LH), and serotonin (5-HT). Data are expressed as percentage of untreated controls. Each treatment resulted in a significant increase in E2 secretion comparing to control. $^{**}p < 0.001$ vs FSH and vs 5-HT; $^{++}p < 0.001$ vs LH and vs 5-HT.

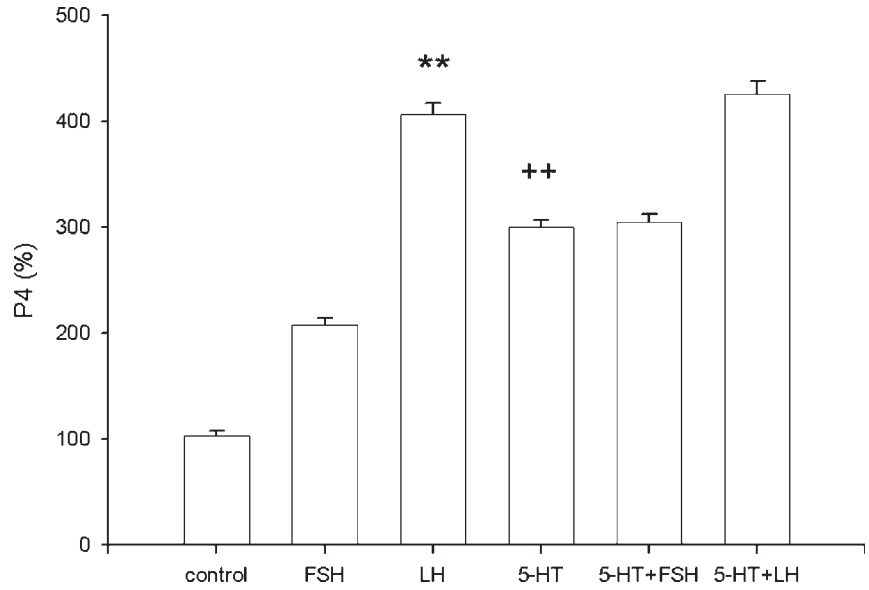

Fig. 2. Progesterone (P4) secretion in human granulosa cell culture treated with follicle stimulating hormone (FSH), luteinizing hormone (LH), and serotonin (5-HT). Data are expressed as percentage of untreated controls. Each treatment resulted in a significant increase in P4 secretion comparing to control. $^{**}p < 0.001$ vs FSH and vs 5-HT; $^{++}p < 0.001$ vs FSH.

Results

E2 Secretion in Cell Culture

FSH, LH, and 5-HT administered alone induced a significant increase in E2 secretion to 248 ± 10%, 228 ± 7%, and 226 ± 9%, respectively, compared to untreated controls ($p < 0.001$). When 5-HT was co-administered with FSH or LH, it further augmented secretion of E2 to values of 321 ± 8% ($p < 0.001$) and 345 ± 14% ($p < 0.001$), respectively, compared to cultures exposed to FSH or LH alone (Fig.1).

P4 Secretion in Cell Culture

FSH, LH, and 5-HT administered alone increased P4 secretion to 207 ± 7%, 406 ± 10% and 299 ± 7%, respectively, compared to untreated cultures ($p < 0.001$). Also, the difference between the increase in LH- and FSH-treated, between LH- and 5-HT-treated, as well as that between 5-HT- and FSH-treated groups proved to be statistically significant ($p < 0.001$). However, co-administration of 5-HT with LH or FSH added no further increase in P4 secretion (Fig.2).

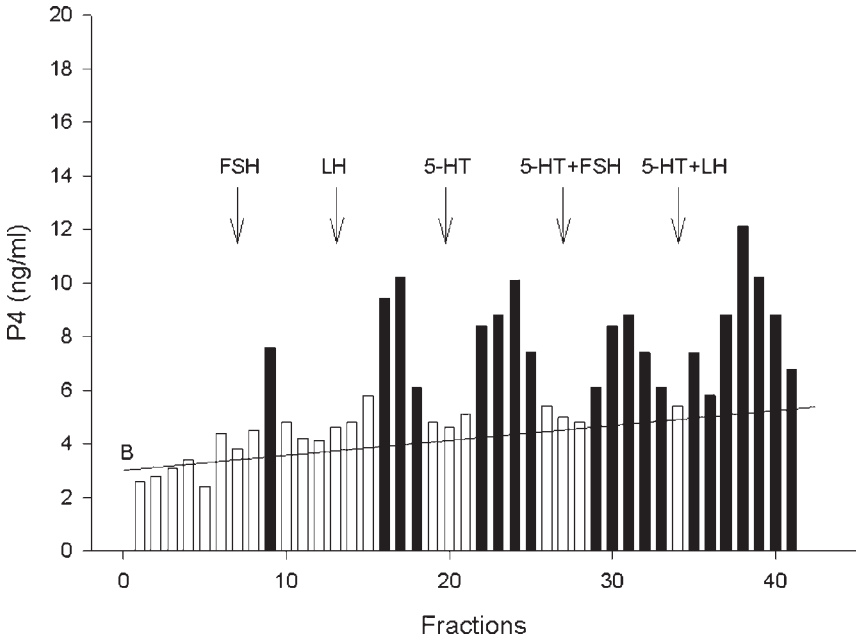

Fig. 3. Progesterone (P4) release in superfused granulosa cell system following administration of follicle stimulating hormone (FSH), luteinizing hormone (LH), and serotonin (5-HT). Arrows represent time of administration. Black bars represent statistically significant peaks. B: baseline.

P4 Secretion in Superfused Granulosa Cell System

FSH, LH, and 5-HT administered as a single challenge induced significant increase in P4 release by 67 ± 14%, 86 ± 0.5%, and 59 ± 6%, respectively. Also, a slight increase in baseline secretion throughout the experiment could be observed. However, when 5-HT was co-administered with FSH or LH, only 23 ± 3% and 36 ± 2% net integral hormone release was detected, respectively. The average peak length showed a 3.5-fold increase when 5-HT and FSH were added in combination, compared to single challenge by FSH. Co-administration of 5-HT with LH resulted in an almost two-fold increase in peak length compared to single challenge by LH (Fig.3, Table 1).

Table 1
P4 Secretion in Superfused Granulosa Cell System

Peak	Range	Max (ng/mL)	Int (ng)	NetInt (ng)	NI/B	NI/T
1	9–9	7.6	15.2	7.32	0.93	0.0143
2	16–18	10.2	51.4	23.98	0.87	0.0468
3	22–25	10.1	69.4	28.76	0.71	0.0561
4	29–33	8.8	73.6	16.92	0.30	0.033
5	35–41	12.1	122.2	35.16	0.40	0.0686

Corresponding peaks are represented on Fig. 3 as black bars. Range: fractions with statistically significant hormone response; Max: highest hormone concentration during response; Int: amount of hormone secreted under the curve of peak; NetInt: net integral amount of secreted hormone under the curve, above baseline; NI/B: ratio of NetInt and baseline; NI/T: ratio of NetInt and total amount of hormone secreted.

Discussion

Data of the present study confirm previous reports on the stimulatory effect of 5-HT on E2 and P4 secretion of human GCs *(4,6,10)*. Also, this article demonstrates a different effect of 5-HT on gonadotropin-induced E2 and P4 secretion in cell culture. Although 5-HT further augmented E2 secretion evoked by FSH or LH, it had no additive effect on gonadotropin-induced P4 secretion in cell culture. Moreover, results demonstrate a modifying effect of 5-HT on the pattern of P4 release induced by FSH or LH in dynamic superfusion system. Co-administration of 5-HT with FSH or LH resulted in less stimulation of P4 while the peak length was markedly prolonged.

Although there is a contradictory report in the literature concerning the stimulatory action of 5-HT on P4 secretion in human GCs *(11)*, a larger body of data demonstrating the stimulatory effect appears to be convincing and is in accord with our findings *(4,6,10)*. Furthermore, in this stimulatory action of 5-HT, the involvement of 5-HT7 receptor type positively coupled to adenylate cyclase has been revealed *(6)*.

The underlying reason for a different response to administration of 5-HT in gonadotropin-stimulated E2 and P4

secretion is yet to be investigated. Jalkanen demonstrated an inhibitory action of E2 on P4 production of GCs *(12)*. This might explain the lack of additive effect of 5-HT on the stimulatory action of both gonadotropins on P4 secretion, when administered along with FSH or LH in cell culture. Also, interaction between gonadotropins- and 5-HT-induced signal transduction pathways might be involved in this mechanism. Because we had no appropriately sensitive method to detect E2 released into the superfusion system, we were unable to examine if there is a distinct response in E2 and P4 release during co-administration of 5-HT with gonadotropins under dynamic in vitro circumstances. The lower amount of P4 release in the superfusion system during the last two peaks is paralleled with longer peak length. On the first sight, it might appear that specific hormone release increases during these challenges. However, an in-depth statistical analysis reveals the fact that, in addition to a new finding of prolonged hormone release, increase in basal hormone secretion contributes to the increased amount of hormone secretion during combined treatments. The reason for the slight elevation of baseline is not clear. The aim of calculating the ratio of net integral hormone release and basal secretion is to determine pure specific response of cells to treatments. To our knowledge, this is the first time that prolongation of effect of gonadotropins by co-administration of 5-HT on P4 release of GCs is demonstrated. An explanation for this finding might be a long-term additive or "priming" effect of prior challenges during the experiment. Also, the underlying reason for the change in the dynamics of P4 secretion might be a modification at receptor level and/or in post-receptor mechanisms of GCs as an effect of 5-HT treatment. Also, it is possible that the significantly changed pattern of P4 release could be attributed to other substances produced by and released from GCs during combined challenges by 5-HT and gonadotropins.

Although there are no data in the literature demonstrating the presence of serotonin containing axons in the ovary, previous observation in immature male rats treated with neurotoxin 5,7-DHT, known for destroying serotoninergic neural elements, suggested the presence of such structures in the male gonad *(13)*. In accord with data on possible ovarian action, the effect of 5-HT on steroidogenesis in the rat testis is well documented *(13)*. Nevertheless, the presence of 5-HT in human follicular fluid samples was previously demonstrated *(14)*, and, in addition to a possible peripheral neural source, blood influx, platelet aggregation, and mediator release in the ruptured follicle turning into corpus hemorrhagicum might provide sufficient amount of 5-HT for paracrine regulation of steroid secretion *(15)*.

In conclusion, in addition to a direct stimulatory effect, 5-HT seems to have a modulatory effect on gonadotropin-induced steroid secretion of granulosa cells. The observed action may have a physiological role in the regulation of hormone production in the corpus luteum.

Materials and Methods

Patients and Treatments

Human granulosa cells were isolated from preovulatory FF aspirated from 36 women (mean age 30.8 yr) undergoing in vitro fertilization. Ovarian stimulation was performed by human menopausal gonadotropin (Humegon; Organon, Oberschleissheim, Germany) under pituitary down-regulation induced by the LH-releasing hormone agonist triptorelin (Decapeptyl; Ferring, Kiel, Germany). Ovulation was induced by a single dose of 10,000 IU human chorionic gonadotropin (hCG, Predalon; Organon), and aspiration of FF was performed 36 h later by ultrasound-guided vaginal puncture avoiding general anesthesia.

Cell Isolation

The detailed method for cell isolation was described in our previous article *(16)*. Briefly, after aspiration, FF was centrifuged and the pelleted GC mass was suspended in fresh McCoy's 5A medium supplemented with 25 mmol/L *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (Hepes), 3 mmol/L L-glutamine (GIBCO Laboratories, Paisley, Scotland), 10^5 IU/L penicillin, and 100 mg/L streptomycin sulfate (Seromed, Berlin, Germany). Granulosa cells were separated from red blood cells by density gradient centrifugation for 20 min at 200–300*g* in 60% Percoll (Pharmacia LKB, Uppsala, Sweden) after the dispersion of cells by repeated pipetting. Viability (ranged between 75% and 90%) of cells was assessed by 0.5% Trypan blue (Serva, Heidelberg, Germany) exclusion. Viable GCs (2×10^5) were cultured in multiwell Linbro tissue culture dishes (1.7 cm diameter; Flow Laboratories, McLean, VA) using 1.0 mL McCoy's 5A medium described above, containing 10% fetal calf serum (FCS; Seromed). They were cultured at 37°C in a humidified atmosphere (95% air + 5% CO_2) for the first 24 h (time 0). Twenty-four hours later (time 1), the GCs were stepped down after vigorous washing, from 10% FCS supplementation to serum-free condition, adding only 0.35% bovine serum albumin (Sigma) to the culture medium. After the second interval of 24 h (time 2) of culture, the medium was removed, and fresh culture medium was added for an additional 48 h. At time 2, 5-HT was added to the wells at a final concentration of 10^{-4} *M*. A solution with a final concentration of 10^{-5} *M* LH (NIH, Bethesda, MD, USA) and 0.1 *M* FSH was added to the wells with or without 5-HT. For each condition three or four replicates per culture were used. At the end of the experiment the supernatant was collected and stored at –20°C until assayed. The viability of the cells was assessed at the end of the incubation period by Trypan blue exclusion.

The superfusion apparatus consisted of a number of 1-mL syringe barrels mounted vertically in a Plexiglas holder that was kept at 37°C by circulating water. A properly greased O-ring prevented the leakage of the circulating water into

the syringe barrels. Each barrel was fitted with plungers at both ends. Holes were drilled in the plungers to accommodate plastic tubing. The lower plunger was covered with a small piece of 300-µm-pore Nylon net to prevent Sephadex G-10 beads from escaping. A multichannel peristaltic pump (Gilson Minipulse type HP-8) that was connected after the superfusion chamber maintained constant flow of McCoy's 5A medium at a rate of 4.0 mL/h. Thus, the system was operated with a negative pressure. Samples were collected with a fraction collector at 15-min intervals into disposable glass tubes. Following the establishment of a stable baseline, FSH (10^{-5} *M*, NIH, Bethesda, MD, USA), LH (10^{-5} *M*, NIH, Bethesda, MD, USA), and 5-HT (100 pmol, Sigma, St. Louis, MO) were pumped into the system at 3 min pulses.

Assays

Estradiol (E2) was measured in the culture media samples by direct RIA kit from Baxter Merz + Dade AG (Dudlingen, Switzerland). The working range of the assay was 0–6000 pmol/L with interassay and intraassay coefficients of variation (CV) of 5.1% and 5.6%, respectively. P4 concentrations were measured using COATRIA ^{125}I-P radioimmunoassay (RIA) without extraction (bioMérieux, Marcy l'Etoile, France). Sensitivity of the assay was 0.15 nmol/L, the intra- and interassay CVs (4.0% and 4.4%, respectively) were in the higher range of the standard curve.

Statistical Analysis

All cell culture experiments were performed in triplicate. In cell culture experiments, one way analysis of variance (ANOVA) was used for statistical analysis. Data are expressed as mean ± SE. Superfusion data were analyzed by a special computer program *(17)*. Using this program, we calculated the basal P4 secretion (baseline, B) and net integral (NI) amount of P4 released (area under the curve above B). Peaks in hormone secretion are expressed as percentage value of basal secretion (NI/B). Superfusion experiments were repeated four times and the most representative one is presented.

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