Stimulation of progesterone secretion by cultured human granulosa cells with melatonin and catecholamines

G. E. Webley, M. R. Luck* and J. P. Hearn

MRC/AFRC Comparative Physiology Research Group, Institute of Zoology, Regent's Park, London NW1 4RY, U.K.; and *Institute of Hormone and Fertility Research, Grandweg 64, 2000 Hamburg 54, F.R.G.

Summary. Granulosa cells, aspirated from the follicles of patients undergoing treatment for in-vitro fertilization, were cultured in serum-supplemented medium. Adrenaline and noradrenaline stimulated a dose-related increase in progesterone secretion with a maximum stimulation at 10^{-5} M, a response that was prevented by the β -antagonist, propranolol. Adrenaline and hCG showed similar characteristics in their stimulation of progesterone secretion but there was no further increase in progesterone when the 2 compounds were added together. Melatonin stimulated progesterone secretion and, like adrenaline, this stimulation was prevented by propranolol. The ability of both adrenaline and melatonin to increase progesterone secretion was dependent on the degree of follicular development, as determined by peripheral oestradiol concentrations, on the day of laparoscopy. These results suggest that adrenaline and melatonin may have a physiological role in modulating luteal function and that melatonin may act by a β -adrenergic-related mechanism.

Keywords: human; granulosa cells; progesterone secretion; melatonin; catecholamines

Introduction

There is now substantial evidence that the primate corpus luteum (CL) requires the luteotrophic support provided by pituitary LH (Knobil, 1973; Ross, 1979; Fraser *et al.*, 1985, 1986). Of less certainty is the role of other hormones which can influence progesterone secretion, either directly or by influencing the actions of known luteotrophic or luteolytic agents, and which may have an important modulating effect on CL function.

These potentially modulatory hormones include melatonin and catecholamines. We have described the ability of melatonin to stimulate progesterone production by human granulosa cells in culture (Webley & Luck, 1986) and by the CL of the marmoset monkey perfused *in vivo* (Webley & Hearn, 1987; Hearn & Webley, 1987).

Catecholamines stimulate progesterone production by the ovary *in vivo* and by CL tissue and granulosa cells *in vitro* in a number of species, including the rat (Ratner *et al.*, 1980; Norjavaara *et al.*, 1982), sheep (Jordan *et al.*, 1978), cow (Condon & Black, 1976; Battista *et al.*, 1987; Luck & Jungclas, 1987, 1988) and cat (Wheeler *et al.*, 1987). It is, however, uncertain whether catecholamines stimulate steroidogenesis in primate CL. In man, adrenaline and noradrenaline have no effect on progesterone secretion by dispersed luteal cells either in short-term incubation or monolayer culture (Richardson & Masson, 1980; Casper & Cotterell, 1984). In the rhesus monkey, catecholamines do not influence cAMP production by homogenates of luteal tissue (Eyster & Stouffer, 1985), although Bennegard *et al.* (1984) have demonstrated the ability of noradrenaline to stimulate progesterone secretion by incubated pieces of human CL, and have postulated a role for catecholamines in determining the life-span of the CL. These authors suggest that the innervation

of blood vessels in the ovary, with the release of noradrenaline, is necessary for the luteolytic action of prostaglandin F-2 α . We have tested the hypothesis that adrenaline and noradrenaline act on the human ovary by studying the response of human granulosa cells cultured *in vitro*.

Materials and Methods

Cell preparation. Granulosa cells were aspirated from multiple preovulatory follicles of women undergoing in-vitro fertilization. The cells used in the present study were from 15 patients in two clinics, The Humana Hospital Wellington, London, U.K., and the Gemeinschaftspraxis Leidenberger & Ptnrs, Hamburg, F.R.G. Multiple follicular development was achieved by stimulation with clomiphene citrate and hMG. The regimen was similar in the two clinics, achieving a pregnancy rate of up to 30%, and has been described previously for the patients in Hamburg (Webley & Luck, 1986). In the Humana Hospital patients received clomiphene (100 mg/day) on Days 2–6 of the cycle and Pergonal on Days 2, 4, 6, 8, and 10 or on Days 5–11 of the cycle. Follicular development was monitored by serum oestradiol concentrations and/or ultrasonography. The patients in both clinics received 5000 i.u. hCG and the follicles were aspirated by laparoscopy 36 h later.

Granulosa cells were recovered from follicular fluid after centrifugation at 250 g for 10 min. The cells from a single patient were suspended in 20–30 ml phosphate-buffered saline (PBS) (Flow Laboratories, Rickmansworth, Herts, U.K.), filtered through a sterile nylon mesh to remove any clumps and separated from erythrocytes on Percoll (Pharmacia, Milton Keynes, Bucks, U.K.) (Webley & Luck, 1986). In brief, the cell suspension was divided between two siliconized glass round-bottom tubes and underlayered with 60% Percoll. 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, resuspended in PBS and washed three times. After the final centrifugation the cells were resuspended in 1 ml PBS and counted by haemocytometer or by DNA estimation, for which a small sample was sonicated. The volume of the cell suspension was adjusted with mixed medium (1:1 of DMEM and Hams F12 (Flow Laboratories)) containing 10% human serum (Gibco, Paisley, U.K.). The serum was inactivated at 56°C for 30 min and sterilized by passage through a 0.2 μ m membrane filter.

Cell culture. Cells were added to plastic 24-well culture plates (Costar (Broadway, Cambridge, MA, U.S.A.) and Nunclon (Gibco)) on Day 0 in 500 μ l medium ($1\text{--}2 \times 10^5$ cells/well in replicates of 4 wells per treatment). The cells from each patient were cultured separately. Treatments were added to the wells on Day 0 in 100 μ l and the final volume was made up to 1 ml per well with culture medium. Adrenaline bitartrate, noradrenaline bitartrate, propranolol and melatonin were purchased from Sigma (Poole, Dorset, U.K.) and diluted in culture medium at the start of each experiment. Melatonin was added in ethanolic solution, with an equivalent quantity of ethanol (20 mM) being added to control wells. The hCG used in the culture treatments was standard CR 121 from NIH (Bethesda, MD, U.S.A.) with a biological potency of 13 450 IU/mg. The culture plates were incubated at 37°C in an humidified incubator (95% air/5% CO₂). Culture media were changed and the treatments renewed every 24 h for up to 6 days and the samples stored at –20°C until assay.

Assays. Progesterone was measured in the culture media samples using two assays, a direct radioimmunoassay (RIA) kit purchased from RSL Inc (Carson, CA, U.S.A.) and a direct enzymeimmunoassay (EIA) as described by Hodges *et al.* (1988). There was a good correlation of progesterone concentrations measured in samples by using the 2 assays over the working range, with a correlation coefficient of 0.94 ($n = 10$, $P < 0.01$; regression slope = 0.78; y intercept = 0.09 pmol/ml). The working range of the RIA was 0.6–65 pmol/ml with inter- and intra-assay coefficients of 13% and 7.6% respectively. The working range for the EIA in culture medium was 0.8–24 pmol/ml with inter- and intra-assay coefficients of 14% and 12% respectively. Samples of uncultured serum-supplemented medium included in each assay showed no detectable progesterone.

Oestradiol-17 β was measured in serum samples and culture medium using a direct RIA kit purchased from Sorin Biomedical (Saluggia, Italy). The working range of the assay was 0.1–2.2 pmol/ml with inter- and intra-assay coefficients of 7.0% and 6.0% respectively.

DNA concentration was measured using the bisbenzimidazole fluorescence method of Labarca & Paigen (1980), with calf thymus DNA (Sigma) as standard. Cell number was calculated according to the relationship 1 μ g DNA = 1.67×10^5 cells.

Statistics. The ability of the treatments to influence cell number was assessed by determining the concentration of DNA after 3 days of culture. As there was no effect of the treatments on cell number the concentrations of progesterone have been normalized for the number of cells present at the start of culture. The data are expressed as mean $\pm$ s.e.m. and analysis of variance (ANOVA) was used to determine statistical significance between control and treatment groups.

Results

The influence of a range of concentrations of adrenaline and noradrenaline (10^{-7} – 10^{-4} M) on the production of progesterone by human granulosa cells is shown in Fig. 1. Both adrenaline and

Fig. 1. Concentrations of progesterone produced by human granulosa cells from 2 patients on Day 3 of culture (i.e. between 48 and 72 h of culture). The cells of one patient were cultured in the presence of a range of concentrations of adrenaline (Ad) and cells from the other patient were cultured with a range of concentrations of noradrenaline (Norad); both treatments were with and without propranolol (prop). Values are mean $\pm$ s.e.m. for 4 replicate wells per treatment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with control values.

noradrenaline stimulated a dose-related increase in progesterone secretion, the maximum stimulation occurring with a concentration of 10^{-5} M. The catecholamines were added from the beginning of the culture and their ability to stimulate progesterone was similar on Days 3 and 4. Incubation of the cells, with the range of concentrations of adrenaline and noradrenaline, in the presence of propranolol (10^{-5} M) prevented the stimulation of progesterone secretion. The response to adrenaline and noradrenaline is shown for cells taken from 2 different patients of 6 giving a similar response.

In a preliminary experiment to determine the dose effect of hCG on progesterone secretion, 1 ng/ml was found to give a submaximal response whereas 10 and 100 ng/ml gave similar maximal responses (data not shown). The stimulation of progesterone production by adrenaline (10^{-5} M) was compared with the response to hCG (100 ng/ml) over 6 days of culture (Fig. 2). There was no effect of either treatment on the first 3 days of culture but adrenaline and hCG stimulated progesterone secretion on Day 4 and thereafter. Control concentrations of progesterone declined from Day 4 to Day 6 but the degree of stimulation (~ 2 -fold) remained constant. The degrees of stimulation were similar for adrenaline and hCG during continuous treatment over the 6 days of culture. Incubation with adrenaline (10^{-5} M) and hCG (10 ng/ml) together and compared with each treatment alone, on Days 3 and 4 of culture, is shown in Fig. 3. There was no further stimulation of progesterone when the two treatments were added together. Adrenaline had no effect on oestradiol-17 β production in this experiment (results not shown).

The degree of stimulation of progesterone production by adrenaline varied between patients (Table 1). Of the 8 patients studied, 6 showed a response to adrenaline whereas there was no effect in 2 patients. A similar variation in response occurred with melatonin (Table 1).

Fig. 2. Concentrations of progesterone from human granulosa cells in culture medium changed every 24 h over 6 days in the presence of adrenaline (Ad, 10⁻⁵ M) and hCG (100 ng/ml). Values are mean ± s.e.m. for 4 replicate wells per treatment. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, compared with control values.

Fig. 3. Concentrations of progesterone from human granulosa cells on Days 3 and 4 of culture in the presence of adrenaline (Ad) (10⁻⁵ M) and hCG (10 ng/ml). Values are mean ± s.e.m. for 4 replicate wells per treatment. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, compared with control values.

Table 1. Concentrations of progesterone produced by granulosa cells from 13 patients on Day 3 or 4 of culture in the presence of melatonin (200 pg/ml) or adrenaline (10^{-5} M) and the percentage difference between the control and treatment wells

Patient no.	Progesterone (nmol. 10^5 cells $^{-1}$.ml $^{-1}$.24 h $^{-1}$)			% Difference
	Control	Melatonin		
1	1.93 $\pm$ 0.33	4.62 $\pm$ 0.32	***	231
2	0.76 $\pm$ 0.03	1.35 $\pm$ 0.11	***	177
3	1.91 $\pm$ 0.13	1.62 $\pm$ 0.16		85
4	3.09 $\pm$ 0.35	3.95 $\pm$ 0.19		128
5	1.31 $\pm$ 0.13	2.11 $\pm$ 0.12	**	161
6	2.20 $\pm$ 0.18	3.28 $\pm$ 0.22	**	149
7	2.56 $\pm$ 0.14	4.36 $\pm$ 0.38	***	183
	Control	Adrenaline		
6	2.20 $\pm$ 0.18	3.12 $\pm$ 0.09	***	142
7	2.56 $\pm$ 0.14	3.98 $\pm$ 0.38	**	156
8	1.82 $\pm$ 0.13	1.99 $\pm$ 0.08		110
9	2.64 $\pm$ 0.22	4.90 $\pm$ 0.10	***	186
10	1.99 $\pm$ 0.11	1.65 $\pm$ 0.06		83
11	1.62 $\pm$ 0.15	2.64 $\pm$ 0.06	***	163
12	1.69 $\pm$ 0.27	2.91 $\pm$ 0.36	*	173
13	2.40 $\pm$ 0.37	4.20 $\pm$ 0.11	***	175

Values are mean $\pm$ s.e.m., $n = 4$.

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

Fig. 4. The degree of stimulation of progesterone is shown as the percentage difference between progesterone concentrations measured in control wells and in (a) the presence of adrenaline (10^{-5} M) and in (b) the presence of melatonin (200 pg/ml), plotted against the concentrations of oestradiol- 17β in the serum of the patients on the day on which the granulosa cells were aspirated.

Fig. 5. Concentrations of progesterone produced by granulosa cells from 2 patients with comparatively high oestradiol-17 β concentrations (Patient a = 6.05 nmol/l, Patient b = 5.33 nmol/l in the presence of melatonin (Mel) (200 pg/ml), adrenaline (Ad) (10^{-5} M), and propranolol (prop) (10^{-5} M). Values are mean $\pm$ s.e.m. for 4 replicated wells per treatment. * P < 0.05, ** P < 0.01, *** P < 0.001, compared with control values.

A significant correlation was found between the degree of stimulation of progesterone by adrenaline ($r = 0.9$, $N = 8$, $P < 0.05$) (Fig. 4a) or melatonin ($r = 0.94$, $N = 7$, $P < 0.01$) (Fig. 4b) and the circulating oestradiol-17 β concentration on the day of follicular puncture. High serum oestradiol concentrations were associated with a large stimulation of progesterone by adrenaline and melatonin. The serum concentration of oestradiol was significantly correlated ($r = 0.93$, $n = 7$, $P < 0.01$) with the total follicular fluid concentration and there was no correlation with the number of follicles. No differences were apparent between cells taken from patients in the two clinics or between the different regimens of clomiphene or hMG stimulation.

The effects of melatonin and adrenaline were further compared in 2 patients (one from each clinic) with comparatively high serum oestradiol concentrations (Fig. 5). In both patients melatonin and adrenaline significantly stimulated progesterone secretion. Melatonin and adrenaline added together (in Patient b) exerted a synergistic effect. Propranolol had no effect on progesterone secretion but it did prevent the stimulations seen with adrenaline and melatonin.

Discussion

This study describes for the first time the stimulatory effects of adrenaline and noradrenaline on progesterone production by human granulosa cells cultured in the presence of serum. The response was similar to that elicited by hCG in both time course and extent. Melatonin stimulated progesterone production and the degree of stimulation by melatonin and adrenaline was dependent on the concentration of oestrogen produced by the follicles at the time of follicular puncture. The response to melatonin and adrenaline was blocked by the β -receptor antagonist, propranolol.

In any study of this nature, the interpretation of results depends on a number of assumptions. Firstly, data for progesterone production have been expressed in terms of concentration per cell number at the start of culture and it has been assumed that the numbers remained the same during the culture period and were not affected by the treatments. This is justified as human granulosa cells cultured in serum do not proliferate or only do so very slowly during the first 6 days of culture (Tapanainen *et al.*, 1987) and the treatments were found to have no effect on cell number after 3 days of culture. Secondly, it is assumed that the cells have undergone a process of luteinization in culture and that the changes in morphology of the cells and the secretion of progesterone are characteristics of the luteinization which occurs in parallel *in vivo* and *in vitro* (Crisp & Channing, 1972; Tureck & Strauss, 1982). A further assumption is that the cells have not been affected by treatment with clomiphene, hMG and hCG beyond their physiological response to gonadotrophins in the normal cycle, as reflected by their production of oestradiol-17 β at the time of collection.

The discrepancy in the reported effects of catecholamines on the human corpus luteum (see 'Introduction') may be in the nature of the preparations studied; dispersed luteal cells are unresponsive, whereas slices are stimulated. Whether this difference reflects absence of intercellular signals which are disrupted by cell dispersion, or the loss of receptors during dispersion, is uncertain. As the granulosa cells studied here appeared to undergo luteinization in culture, it is possible that the ability of the cells to respond to catecholamines resulted from the process of luteinization.

Certain characteristics of the stimulation of progesterone production by adrenaline and hCG were similar. The human granulosa cell in the present study is therefore like the rat luteal cell (Harwood *et al.*, 1980) in that both cells respond to hCG and adrenaline but there is no additive effect on progesterone secretion. It was suggested from studies in the rat that catecholamines and hCG act via independent receptor systems to stimulate a common pool of adenylate cyclase (Richardson, 1986). In contrast, the response of human granulosa cells to the β -adrenergic agonist, isoproterenol, and to hCG in the study of Hillensjo *et al.* (1985) was rather different with isoproterenol having no effect alone but enhancing the action of hCG. The inability of the granulosa cells to respond before Day 4 of culture agrees with previous studies in which granulosa cells taken from the follicles of patients exposed to a preovulatory injection of hCG could not be stimulated with hCG during the first 2–4 days of culture (Schmidt *et al.*, 1984; Hillensjo *et al.*, 1985; Tapanainen *et al.*, 1987). Tapanainen *et al.* (1987) suggest that the cells were already maximally stimulated *in vivo* by hCG and could not be stimulated further *in vitro*. Since the cells in culture were continually exposed to hCG the attainment of response by Day 4 of culture may instead represent a maturation process. This could be similar to the changes which occur to the corpus luteum *in vivo*; hCG had no effect on progesterone production by early human luteal tissue (up to 4 days after the LH peak) whereas it stimulated production by mid-luteal tissue (Vega *et al.*, 1987).

Granulosa cell responses to adrenaline and melatonin correlated with circulating oestradiol concentrations which were indicative of total follicular oestradiol production. High concentrations of oestradiol in follicular fluid have been shown to be associated with mature follicles containing oocytes which have a greater ability to fertilize *in vitro* (McNatty *et al.*, 1979; Bomsel-Helmreich *et al.*, 1979). In the rat ovary, β -adrenergic receptors change with the stage of the oestrous cycle and are correlated with serum oestradiol concentrations (Jordan, 1981). It therefore appears likely that oestradiol stimulates the development of β -adrenergic receptors as an important part of follicular function.

Webley & Leidenberger (1987) have shown an increase in the circadian output of melatonin during the luteal phase of the menstrual cycle and demonstrated the ability of melatonin to increase the production of progesterone by the corpus luteum of the marmoset monkey perfused *in vivo* (Webley & Hearn, 1987; Hearn & Webley, 1987). The mechanism by which melatonin acts centrally or peripherally is as yet unknown. In the present study the responses of human granulosa cells to adrenaline and melatonin were similar in that they were both dependent on the degree of follicular development, as represented by peripheral oestradiol concentrations. In addition the β -receptor antagonist propranolol blocked the stimulation of progesterone by both adrenaline and melatonin.

Melatonin, at physiological concentrations, increases the binding of β -adrenergic agonists by pineal tissue *in vitro* (Sweat, 1986), perhaps indicating the mechanism by which it acts, but melatonin was also active in stimulating progesterone secretion by human granulosa cells in the absence of added catecholamines, suggesting an additional mechanism. The inhibition of the action of melatonin by propranolol indicates that melatonin may be acting on a β -receptor.

The ovary has a rich autonomic innervation (Burden, 1985) and catecholamines may also reach the ovary via the peripheral circulation. Melatonin is present in the follicular fluid of preovulatory follicles and at higher concentrations than in the corresponding peripheral circulation (Brzezinski *et al.*, 1987). Propranolol infused *in vivo* in the ovary or the peripheral circulation resulted in decreased circulating progesterone concentrations during the luteal phase in the anaesthetized cat (Wheeler *et al.*, 1987) and the sheep (Wheeler *et al.*, 1988). These results suggest a dependence, to a degree, of progesterone on the sympathetic nervous system. The results presented here further suggest that catecholamines and melatonin may have a physiological role in modulating luteal function.

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