Luteinization Factor-Stimulated Steroidogenesis in Porcine Granulosa Cells

M Ježova, S Vršanska and J Kolena

Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlarska 3, 833 06 Bratislava, Slovakia

Abstract. Luteinization stimulator (LS), an intrafollicular compound of preovulatory (5–8 mm) follicles, increased both the basal and gonadotropins-stimulated production of progesterone by immature (1–3 mm) granulosa cells The mechanism by which LS enhance steroidogenesis was investigated by studying the modulation of progesterone biosynthesis from exogenous cholesterol and pregnenolone in cultured porcine granulosa cells in serum-free medium Progesterone production by cultured granulosa cells was stimulated by FSH, while treatment with 22-OH-cholesterol further enhanced the gonadotropin action The activity of LS was found in cell conditioned media obtained after 3-day cultivation of preovulatory granulosa cells Conversion of 22-OH-cholesterol into progesterone by granulosa cells isolated from small follicles was significantly stimulated in the presence LS in culture media Also, progesterone production by granulosa cells in the presence of pregnenolone was increased considerably Concomitant treatment with LS led to a further augmentation in progesterone synthesis Endogenous formation of pregnenolone was inhibited by aminoglutethimide Thus, LS enhancement of progesterone production in cultured porcine granulosa cells is associated with an increase in the activity of cytochrome P450 cholesterol side-chain cleavage and 3β hydroxysteroid dehydrogenase enzymes

Key words: Luteinization stimulator — Progesterone biosynthesis — Granulosa cell culture — Steroidogenesis

Introduction

Luteinization stimulator (LS) is a nonsteroidal intraovarian factor which modulates the regulatory effects of gonadotropins on the luteinization process of granulosa cells (Ledwitz-Rigby and Rigby 1979, Channing et al 1982) LS has been partially purified from follicular fluid isolated from large preovulatory follicles and an apparent molecular weight of about 28 000 Da has been estimated (Kolena and Channing

Correspondence to Miroslava Ježova, Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlarska 3, 833 06 Bratislava, Slovakia
E-mail ueenjez@savba sk

1985). A factor described as a heat-labile protein is produced preferentially by granulosa cells isolated from large porcine follicles (Šeböková and Kolena 1987). Follicular fluid from large follicles has been reported to enhance basal as well as FSH-stimulated progesterone secretion. The same stimulatory effects in immature granulosa cell culture were observed with partially purified stimulatory activity of follicular fluid (Kolena and Channing 1985). Similar steroidogenic-inducing protein was isolated from human follicular fluid (Khan et al. 1990). This factor stimulated corticosteroid production by adrenal cells, testosterone production by Leydig cells, and formation of progesterone by human granulosa-lutein cells under the basal condition or after hCG treatment.

Steroidogenesis in porcine granulosa cells is controlled by many factors. One of them is the LS occurring in follicular fluid. A stimulatory effect on progesterone secretion, comparable with the activity of follicular fluid was observed in conditioned media of porcine granulosa cells (Danišová and Kolena 1992). It is generally accepted that the rate-limiting step in the pathway concerned with steroid hormone biosynthesis is the conversion of cholesterol to pregnenolone. It is at this locus that gonadotropins are thought to act. The intracellular actions of ovarian stimulatory factors are probably mediated in a similar manner. In the present study, therefore, the stimulatory effect of FSH and LS on conversion of cholesterol and pregnenolone into progesterone by granulosa cells isolated from small porcine follicles was examined.

Materials and Methods

Materials

Porcine FSH (2039 IU/mg) was generously supplied by Dr. Parlow, NIAMDD, NIH, Bethesda, U.S.A. Hams'F-10 medium and transferrin were purchased from Serva, Heidelberg, Germany and all other chemicals were from Sigma, U S.A.

Methods

Porcine ovaries were collected in ice-cold physiological saline plus antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml) immediately after slaughter from a local abattoir and transported to the laboratory. Granulosa cells were aspirated from large antral (5–8 mm) follicles (LGC) or small (1–2 mm) follicles (SGC), washed three times in Medium 199 with Earle's Salts and Hepes buffer (25 mmol/l) and dispersed in Medium 199 supplemented with L-glutamine (1 mmol/l), penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). Incubation of granulosa cell suspension was carried out in 24-well dishes (with well diameter of 15 mm) at 37°C in 5% CO_2 – 95% air (Kolena and Channing 1985). The density and cell viability were determined in a hemocytometer by trypan blue exclusion. The cell viability ranged from 80% to 90%. Cells were incubated during a 72 hour period at a density of 1 1.5×10^6 viable cells/0.5 ml in culture medium supplemented with 10% fetal calf serum. At the end of the incubation period,

the LGC conditioned media (CM) containing LS as well as the SGC media were collected and assayed for progesterone.

The conversion of 22-OH-cholesterol and pregnenolone into progesterone by granulosa cells was carried out by incubation in serum-free medium following the method of Baranao and Hammond (1986) with slight modifications. Serum-free medium consisted of a 1 : 1 mixture of Dulbecco's modified Eagle's medium and Ham's F-10 supplemented with transferrin (5 μg/ml), penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). Granulosa cells were cultured in the same experimental conditions as described above, at a density of 1–1.5 $\times$ 10^6 viable cells/0.5 ml. LGC conditioned media (CM) containing LS used in these experiments were prepared by incubation of granulosa cells in serum-free conditions as described above. Granulosa cells mitochondria were isolated essentially by the method of Cok et al. 1997.

The bioactivity of conditioned media was determined by adding 125 μl (25%) of conditioned media to granulosa cells isolated from small follicles (Danišová and Kolena 1992). After 72 h incubation, the progesterone production was determined. The progesterone values in SGC media were corrected for the amount of progesterone present in the CM media. The changes in the progesterone production by SGC reflect the changes in the amount of LS present in the tested LGC conditioned media.

At the end of incubation period, SGC and LGC media were collected for progesterone determination. Level of progesterone in medium was determined by the [^{125}I]-progesterone radioimmunoassay without extraction (Kolena et al. 1977) using a specific antiserum against 11-OH-progesterone succinyl-BSA (kindly donated by Dr. Tománek, Research Institute of Animal Production, Prague, Czech Republic).

Data were analysed *via* analysis of variance (ANOVA) and Bonferroni post test. Significance was assumed when $p < 0.05$.

Results

To avoid effect of cholesterol and lipoproteins present in the serum, the conversion of 22R-OH-cholesterol and pregnenolone to progesterone by granulosa cells was carried out in serum-free medium. Cholesterol hydroxylated in the C-22 position is readily taken up by the steroidogenic cells. The addition of FSH (1 μg/ml) or 22R-OH-cholesterol (5 μg/ml) to the culture of SGC significantly ($p < 0.05$) increased synthesis of progesterone. 22R-OH-cholesterol in the used concentration significantly ($p < 0.001$) stimulated FSH-induced progesterone synthesis by granulosa cells (Fig. 1).

In previous studies we have shown that granulosa cells in culture are able to produce stimulatory activity which meet the criteria for LS found in follicular fluid from preovulatory follicles and its production is markedly stimulated by insulin (Danišová and Kolena 1992). The ability of LGC to produce LS in serum-free medium is shown in Fig. 2. The LS secretion was evaluated *via* determination of SGC progesterone production after addition of 25% LGC-conditioned media

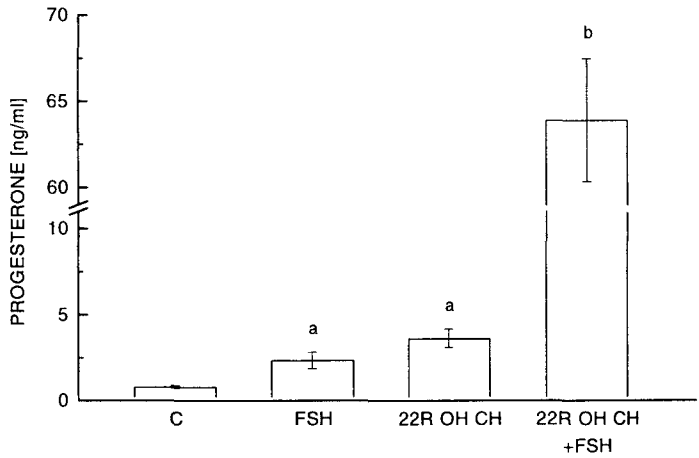

Figure 1. Progesterone production by granulosa cells isolated from small follicles (SGC) cultured in serum-free medium SGC were cultured for 72 h with or without FSH (1 μg/ml) or 22R-OH-cholesterol (22R-OH CH, 5 μg/ml) The values are the means ± S E M of 4 estimations Significant differences ($p < 0\ 05$) were observed between values indicated by different letters and/or controls

(CM) to the culture of SGC CM and CM-I (formation LS in the presence of insulin) media significantly ($p < 0\ 01$) increased production of progesterone by granulosa cells Addition of culture media to granulosa cells in the presence of 22R-OH-cholesterol further enhanced progesterone secretion ($p < 0\ 001$) The cleavage of the cholesterol side-chain by the cytochrome $P450_{scc}$ complex occurs in the inner mitochondrial membrane Fig 3 illustrates conversion of 22R-OH-cholesterol to progesterone by mitochondrial fraction of granulosa cells SGC were cultured in the presence or absence of conditioned media with LS (CM-C) Synthesis of progesterone by mitochondrial fraction obtained from granulosa cells preincubated in the presence of LS was significantly ($p < 0\ 05$) higher in comparison with controls without LS

Finally, we have investigated the effect of LS on conversion of pregnenolone to progesterone by 3β hydroxysteroid dehydrogenase Endogenous formation of pregnenolone was blocked by aminoglutethimide (cytochrome $P450_{scc}$ inhibitor) As shown in Fig 4, progesterone accumulation in the presence of pregnenolone was increased significantly ($p < 0\ 001$) Concomitant treatment with conditioned media (CM-C or CM-I) led to a further augmentation in progesterone synthesis ($p < 0\ 01$)

Discussion

Granulosa cells within the follicle undergo maturation changes during follicular

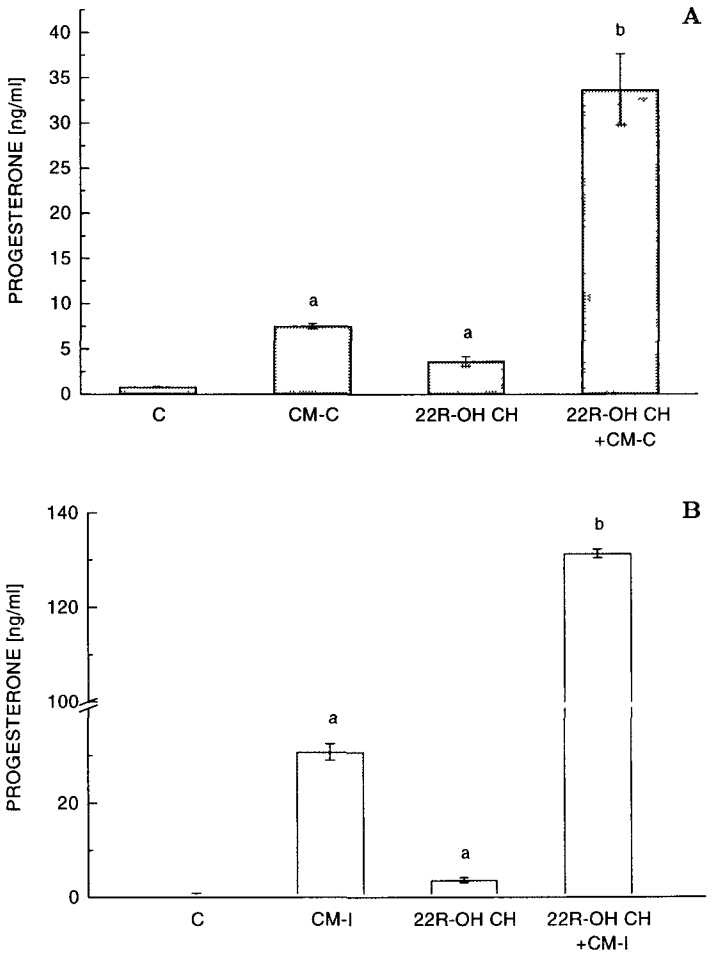

Figure 2. Effect of conditioned media (CM) of granulosa cells isolated from large follicles on progesterone synthesis by SGC CM were collected after 3-day incubation of granulosa cells from preovulated follicles in serum-free medium at the absence (A, CM-C) or presence of insulin (B, CM-I, 5 mg/ml) For details, see legend to Fig 1

differentiation leading to luteinization. FSH and LH are the major regulators of these changes. The action of these pituitary gonadotropins is modulated by other peptide hormones, neurotransmitters, steroid hormones, growth factors, components of extracellular matrix and some nonsteroidal factors produced locally by the ovary (Channing et al. 1982). A luteinization stimulator (LS) which stimulates the luteinization process of granulosa cells is involved in the control of maturation and development of ovarian follicles. LS was demonstrated in follicular fluid isolated from preovulatory follicles of various species, while a low stimulatory ac-

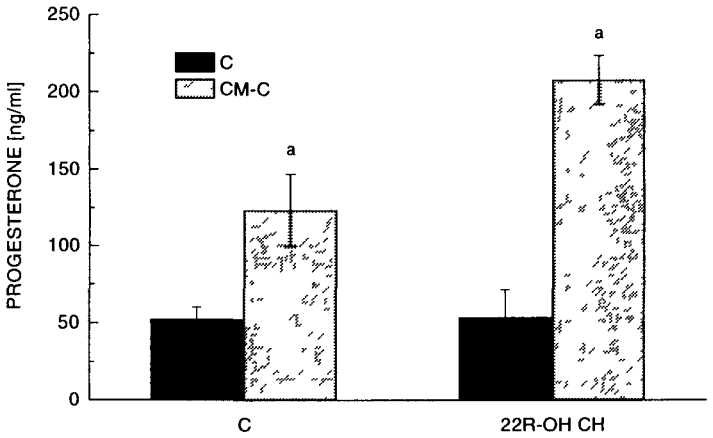

Figure 3. Effect of CM on synthesis of progesterone by mitochondrial fraction of small granulosa cells SGC were cultured 72 h in a medium supplemented with serum at the absence or presence of CM-media (CM-C) Mitochondrial fraction of SGC was incubated at 37 °C for 60 min For details, see legend to Fig 2

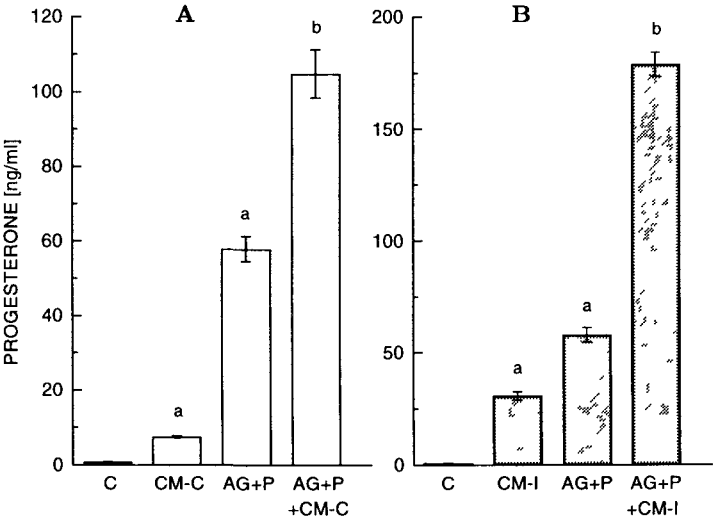

Figure 4. Effect of CM on the conversion of pregnenolone to progesterone by granulosa cells isolated from small follicles SGC were cultured for 72 h in serum-free medium with or without CM-C, CM-I, pregnenolone (P, 10^{-5} mol/l) or aminoglutethimide (AG, 0 25 mmol/l) For details, see legend to Fig 2

tivity was found in follicular fluid from small or atretic follicles (Younglai 1972, Ledwitz-Rigby and Rigby 1979; Channing et al. 1982; Kolena and Channing 1985)

It was also found that spent conditioned media from porcine granulosa cells culture contain a factor which meet the criteria for LS (Šeböková and Kolena 1987). The mechanism whereby LS stimulates the process of luteinization of granulosa cells is not yet clear. It was suggested that stimulatory effect of follicular fluid on FSH-induction of LH/hCG receptors may be involved in the regulation of steroidogenesis by granulosa cells (Kolena and Channing 1985). The absence of alterations in cAMP synthesis by granulosa cells treated with follicular fluid (Kolena et al. 1993) allow us to suggest that LS probably does not operate through the adenylylcyclase signalling system. Follicular fluid was shown to enhance cGMP levels in granulosa cell cultures (Kolena et al. 1993). However, the physiological relationship between cGMP synthesis and steroid production remains to be further investigated.

Biosynthesis of progesterone in granulosa cells is dependent on the availability of free cholesterol in the cells. Cholesterol obtained by the cell is transported to the inner mitochondrial membrane where the cleavage of the cholesterol side-chain by the cytochrome P450 side-chain cleavage enzyme ($P450_{scc}$) complex occurs. The conversion of cholesterol to pregnenolone is the rate-limiting step in steroidogenesis and is the point at which stimulation of progesterone synthesis by tropic hormones is thought to occur. Gonadotropins have been shown to stimulate granulosa cell synthesis of $P450_{scc}$ and electron transport protein adrenodoxin by increasing the levels of their mRNA (Urban et al. 1991; Trzeciak et al. 1986). The present experiments demonstrate the stimulatory effect of FSH and LS on conversion of cholesterol to progesterone in porcine granulosa cells. Although cholesterol is the intracellular substrate used for progesterone synthesis the uptake of this molecule by cells is limited. Cholesterol derivative 20-OH-cholesterol has been helpful in studying steroidogenesis. This cholesterol analog has been shown to freely diffuse into cell and mitochondria to act as a substrate for conversion to pregnenolone by steroidogenic cells (Hall and Young 1968). Results of the present studies indicate that granulosa cells in serum-free medium are able to respond to FSH and LS stimuli with increasing progesterone production. Steroidogenic cells can obtain cholesterol either from serum lipoproteins or by *de novo* synthesis, and in the ovary lipoprotein cholesterol is the main substrate for synthesis of progesterone. Experiments carried out in serum-free medium provide an opportunity for studying the regulation of steroid biosynthesis in the absence of serum lipoproteins. In cell culture lacking serum lipoproteins, granulosa cells effectively utilize incorporated cholesterol for progesterone biosynthesis.

Pregnenolone diffuses from the mitochondria back to the smooth endoplasmic reticulum where it is converted to progesterone by 3β hydroxysteroid dehydrogenase . The stimulatory effect of FSH on the activity of 3β hydroxysteroid dehydrogenase has been documented in cultured granulosa cells. The present study further indicates that LS treatment of granulosa cells increase biosynthesis of progesterone from pregnenolone. Although 3β hydroxysteroid dehydrogenase is not considered a rate-limiting step for steroidogenesis, it plays a key role in the synthesis of progesterone in luteal cells. Therefore, the LS-stimulated progesterone production in cultured porcine granulosa cells appears to be controlled by either cholesterol avail-

ability, activity of $P450_{scc}$ enzyme complex and 3β hydroxysteroid dehydrogenase or a combination of these mechanisms

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