Vol. 151, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1988 Pages 163-169

STIMULATION OF AROMATASE ACTIVITY IN IMMATURE PORCINE LEYDIG CELLS BY FIBROBLAST GROWTH FACTOR (FGF)

James I. Raeside*, Marie-Claude Berthelon, Pascale Sanchez and José M. Saez

INSERM U307, Hopital Debrousse, 69322 LYON Cedex 05, France

Received January 20, 1988

SUMMARY - The effects of fibroblast growth factor (FGF) on testicular aromatase activity has been studied using primary cultures of porcine Leydig cells. After culture for 3 days in the absence or presence of FGF, the ability of the cells to produce estrogen was examined in a 4h-test period in which either (a) hCG (10^{-9} M) or (b) androstenedione ($3x10^{-6}$ M) was added to the medium. FGF produced a 3- to 20-fold increase in estrogen formation from endogenous or exogenous substrate during the test period, in spite of a marked decrease ($\simeq$ 60%) in [^{125}I]-hCG binding and no significant change in testosterone concentration. Stimulation of estrogen secretion by FGF was dose-($ED_{50} \simeq 2$ ng/ml) and time-dependent, the first and maximal effects were observed after 12h and 48h, respectively. Preliminary tests with several other factors (insulin, EGF, TGF-β, FSH and hCG) showed that hCG alone directly stimulated aromatase activity. From these findings a role is suggested for FGF as a paracrine/autocrine agent in the control of estrogen secretion by Leydig cells.

Although Luteinizing Hormone (LH) is the main hormone controlling Leydig cell steroidogenesis, there is increasing evidence that the testes produce polypeptides which locally can modulate gonadal function (1-4). In a recent study of the effects of several factors on pig Sertoli cell growth and function it was shown that basic fibroblast growth factor (FGF) was the most active (5). Since FGF appears to stabilize the phenotypic expression of differentiated cells, especially mesoderm-derived cells in culture (6), it was of interest to examine the effects of this factor on Leydig cells. A remarkable feature of steroidogenesis in pig testes is the high rate of estrogen secretion by the Leydig cells (7). In the present study we report evidence of a specific effect of FGF on aromatase activity, using a primary culture of pig Leydig cells maintained in a chemically defined medium.

* Present address: Dept. of Biomedical Sciences, University of Guelph, Guelph, Ontario, Canada N1G 2W1

To whom correspondence and reprint requests should be addressed.

0006-291X/88 $1.50

MATERIALS AND METHODS

Reagents and hormones

Bovine brain basic FGF was purified as described by Gospodarowicz et al (8) using heparin-Sepharose affinity chromatography. Transforming growth factor (TGF-β) purified from human platelets was a generous gift from Dr. A.B. Sporn (NCI, Bethesda, MD). Epidermal growth factor (EGF) from mouse submaxillary gland was prepared as described by Savage and Cohen (9). Human chorionic gonadotrophin (hCG, Pregnyl) was obtained from Organon, and purified hCG (batch CR-121) was a gift from Dr. R.E. Canfield (New York). Porcine follicle stimulating hormone (NIH-FSH-P_2) was provided by NIADDK, National Pituitary Agency. Collagenase was purchased from SERVA, Heidelberg. Powdered media were from Island Biological Co., Grand Island, NY. Porcine insulin, human transferrin, vitamin E, Hepes and soybean trypsin inhibitor were obtained from Sigma. Non-radioactive steroids were supplied by Steraloids, while [^{3}H]-estrone sulfate and [^{3}H]-testosterone were from New England Nuclear.

Porcine Leydig cell preparation and culture

Preparations of dispersed Leydig cells were made from immature porcine testes at 3-4 weeks after birth, with collagenase treatment and a discontinuous Percoll gradient as described previously (10), with slight modifications. Cells were plated at a density of 0.8-1.0x10^6 cells per well in multiwell culture dishes (Flow Labs) and cultured in 1.5 ml of Dulbecco's modified Eagle's medium (DMEM): Ham's F12 (1:1) containing 0.2% fetal calf serum (FCS) at 34°C in a humidified atmosphere at 5% CO_2 in air for >48h. Then the medium was removed and cells were cultured in 1.0 ml DMEM/F12 medium, without FCS, for 72h with supplements given as indicated in RESULTS. All factors were added daily in 50 µl of medium.

Aromatase activity

To assess the effect of treatment on aromatase activity, two approaches were taken. Medium was removed after 72h of treatment and culture was continued for a further 4h with either (a) 3x10^{-6} M androstenedione or (b) hCG (10^{-9} M) in 1 ml of medium. At the end of the 4h-incubation test, media were removed and stored at -20°C until assayed for steroids. Cellular protein was measured by the Lowry method (11). In some experiments [^{125}I]-hCG binding was determined as described previously (10,12).

The production of estrone sulfate was determined by radioimmunoassay (RIA) with a direct method on media samples using an antiserum and procedures described previously for media from incubations of mature porcine Leydig cells (13). In the present study it was found that >90% of the estimated estrogen appeared in the aqueous phase after ether extraction; and, therefore, RIA was performed with [^{3}H]-estrone sulfate (E_1S) as the radiolabelled ligand in the assay. Results are expressed as E_1S (ng/mg of protein) formed in the 4h-period. For comparison, the testosterone content of the medium was also measured by RIA (14) in some instances where androstenedione had not been added as substrate to the medium.

Statistical analyses

The significance of differences between groups was assessed using Student's t-test or by analysis of variance.

RESULTS

To determine whether some growth factors have an effect on estrogen production, Leydig cells were cultured in their presence for 72h, and then

TABLE 1. EFFECTS OF VARIOUS FACTORS ON PIG LEYDIG CELL FUNCTIONS

Pretreatment			hCG stimulated secretion	
	[^{125}I]-hCG bound	E_1S from Δ^4	E_1S	Testosterone
Insulin (10 μg/ml)	182±18[a]	110± 9	146± 11[a]	220±25[a]
FGF (50 ng/ml)	37± 4[a]	1180±440[a]	940±420[a]	124±15
Insulin + FGF	40± 4[b]	1230±490[b]	1090±340[b]	240±15[a]
Insulin + TGF-β (1 ng/ml)	26± 4[b]	88± 8	97± 7	42± 5[b]
Insulin + hCG (10^{-10}M)	6± 1[b]	850±180[b]	220± 45[b]	40± 6[b]
Insulin + FSH (50 ng/ml)	102± 8	110± 12	114± 11	98± 7

Purified pig Leydig cells were cultured for 72h in the presence of various factors. At the end of the incubation some dishes were used to measure the binding of[^{125}I]-hCG, whereas others were incubated with either androstenedione (Δ^4) (3.10^{-6}M) or hCG (10^{-9}M) for 4h and then the amount of estrone sulfate (E_1S) or testosterone (T) in the medium was measured. The results expressed as per cent of values for cells pretreated with medium alone without insulin (control), are the mean ± SEM of four wells for each pretreatment from three to six independent experiments. The E_1S production by control cells incubated with androstenedione or hCG was 7.6 ± 1.6 and 4.5 ± 0.4 ng/ml protein/4h, respectively.

a : $p < 0.05$ compared to control

b : $p < 0.05$ compared to insulin 10 μg/ml

exposed to a saturating concentration of androstenedione or stimulated with hCG for 4h. As seen in Table 1, FGF was the only factor, apart from hCG, which caused a marked increase in estrogen formation. Stimulation by FGF in 10 different experiments ranged from 350 to 2180% of control values and accounted for the large SEM for E_1S. Increased estrogen secretion occurred in the face of a decline in [^{125}I]-hCG binding and no apparent difference in testosterone production from that of control cells. Neither TGF-β nor FSH pretreatment resulted in an elevation in E_1S levels on subsequent testing (Table 1); nor did EGF (1-50 ng/ml) have an effect on Leydig cell aromatase (data not shown).

The presence of insulin in the medium resulted in marginal effects on the responses by the cells, when compared with control or FGF-treatment alone. For further examination of a possible interaction on estrogen production, an experiment was made with increasing doses of FGF (0.5-10 ng/ml) and insulin (5 μg/ml). The results of Table 2 show that the stimulation of E_1S formation by

TABLE 2. EFFECTS OF FGF WITHOUT OR WITH INSULIN ON LEYDIG CELL ESTROGEN SULFATE (E1S) PRODUCTION (NG/MG PROTEIN/4H)

	E_1S from Δ^4		E_1S from hCG stimulation	
FGF (ng/ml)	- Insulin	+ Insulin	- Insulin	+ Insulin
0	5 ± 0.7	6 ± 0.3	2.5 ± 0.4	4.1 ± 0.6[a]
0.5	10 ± 1.1	11 ± 1.4	5.9 ± 0.6	8.2 ± 0.8[a]
1	16 ± 2.1	15 ± 1.3	11.2 ± 1.2	14.4 ± 1.2[a]
2.5	23 ± 2.2	22 ± 1.9	17.4 ± 2.2	20.1 ± 2.2[a]
5	32 ± 3.1	30 ± 2.1	23.2 ± 1.9	28.1 ± 2.2[a]
10	37 ± 2.5	36 ± 3.1	26.4 ± 2.1	31.2 ± 2.4[a]

Purified pig Leydig cells were cultured for 72h in the absence or presence of insulin (5 µg/ml) without or with the indicated concentrations of FGF. FGF was added every other day. At the end of the preincubation, some cells were incubated with Δ^4-androstenedione (3.10^{-6}M) whereas others were incubated with hCG (10^{-9}M) for 4h. This incubation medium did not contain insulin. Estrone sulfate (E_1S) formed was measured as indicated in Methods. The results are mean ± SEM of three different experiments in quadruplicate.
a : $p < 0.05$ compared to the value without insulin of the same experimental protocol.

FGF is dose-dependent, and is apparently unaffected by the presence of insulin when aromatase activity was evaluated with exogenous substrate. However, insulin enhanced slightly but significantly the effects of FGF on E_1S formation when tested by acute hCG stimulation.

The dose-responses for FGF and hCG on aromatase activity were investigated next (Fig. 1). A maximal effect of FGF was observed between 5 and 10 ng/ml ($ED_{50} \simeq 2$ ng/ml), whereas the maximal response of hCG was seen at 10^{-10} M ($ED_{50} \simeq 10^{-11}$ M). The time-course effects on aromatase activity were somewhat different for the two agents (Fig. 2). FGF required more than 12h to elicit a stimulation, with a maximal response after 48h exposure of Leydig cells at 50 ng/ml (Fig. 2) or 2 ng/ml (data not shown). On the other hand, hCG induced a small but significant increase within 4h, and a further increase between 12 and 48h.

DISCUSSION

These findings demonstrate a totally new activity for basic FGF and add to the growing list of its actions on the phenotypic expression of many types of

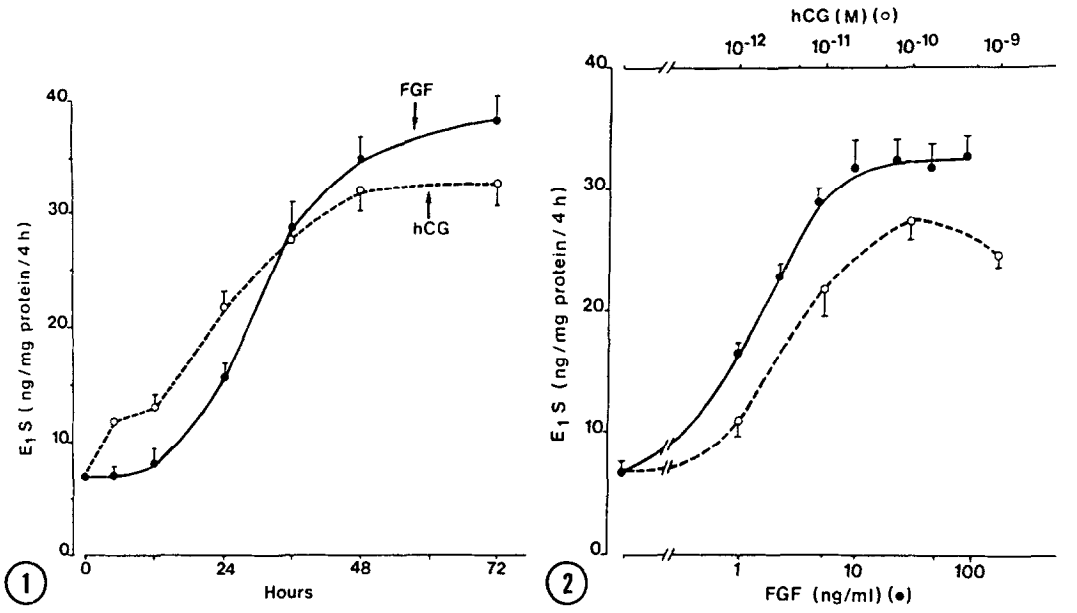

Figure 1. Effects of increasing concentrations of hCG and FGF on Leydig cell aromatase activity. Cells were cultured for 72h with the indicated concentrations of hCG (O) or FGF (●). Then the medium was removed, the cells were washed and incubated in fresh medium, without the factors, in the presence of 3.10^{-6}M androstenedione for 4h. E_1S in the medium was measured and the results are the mean ± SD of two experiments done in quadruplicate.

Figure 2. Time-course effects of hCG and FGF on Leydig cell aromatase activity. Cells were incubated with FGF (50 ng/ml) or hCG (10^{-10}M). At the indicated times, the medium was removed, the cells washed and then incubated in fresh medium, without the factors, in the presence of 3.10^{-6}M androstenedione for 4h. The amount of E_1S in the medium was measured and the results are the mean and the range of four dishes.

differentiated cells. This study shows that FGF clearly enhances the production of estrogens by Leydig cells in culture, and that conversion of androstenedione to estrogen is stimulated in both a dose- and time-dependent manner. A limited number of other factors (insulin, FSH, EGF and β-TGF) were tested but FGF alone was found to stimulate aromatase activity. Thus FGF appears to be the only factor known at present, apart from LH/hCG, which can independently increase estrogen production in Leydig cells in a specific manner.

Since FGF has been identified in the testis (15), its action as a possible paracrine and autocrine agent in the regulation of testicular function deserves consideration. In this regard, it has been shown recently that FGF appears to be not only the most potent known mitogenic factor for Sertoli cells but also to stimulate their secretion of plasminogen activator activity (5). Furthermore, there is evidence now from this laboratory that cultured Sertoli

cells secrete a FGF-like substance (O. Avallet and J.M. Saez, unpublished results).

It is clear also that there was no synergistic effect between FGF and insulin on Leydig cell aromatase activity. In contrast, FGF and somatomedin C/insulin-like growth factor act synergistically on Sertoli cell growth and function (5). Insulin at micromolar concentrations was able to maintain a level of [^{125}I]-hCG binding comparable to that of cells in control media. This would account for the increased E_1S secretion over basal and over FGF-stimulated production when insulin was included in the media, presumably from increased amounts of endogenous precursors formed during the 4h-test period with hCG, in each instance. No significant differences were noted with insulin, however, in the more direct assessment of aromatase activity with androstenedione, or from [^{3}H] water released from [1β, 2β-^{3}H] androstenedione (data not shown), added to the media.

The time-course and the maximal effects of FGF were different from those of hCG, suggesting that the mechanisms involved in the stimulation of aromatase activity by the two factors were different. In favour of this hypothesis is the fact that FGF has no effect on cAMP and testosterone production by the pig Leydig cells (unpublished results). Also, FGF and hCG pretreatment greatly reduced [^{125}I]-hCG binding but without an apparent effect on the stimulatory action of FGF on E_1S formation from either endogenous or exogenous substrate. Further studies are necessary to explore the interrelationship of these two factors which alone are known to enhance aromatase activity of Leydig cells, one as an endocrine agent (LH/hCG) and the other (FGF) as a possible paracrine/autocrine modulating agent. Studies on the regulation of estrogen biosynthesis in vitro in human adipose (16,17) and granulosa cells (18) suggest a complex and multifactorial control of estrogen secretion by Leydig cells also.

ACKNOWLEDGEMENTS

This work was supported by grants from Institut National de la Recherche Agronomique et la Fondation pour la Recherche Medical Francaise. The authors thank Ms. J. Bois and K. Best for their expert secretarial assistance.

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