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BASIC FIBROBLAST GROWTH FACTOR INHIBITS Δ^5-3β-HYDROXYSTEROID DEHYDROGENASE-ISOMERASE ACTIVITY IN CULTURED IMMATURE LEYDIG CELLS

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SUMMARY: Basic fibroblast growth factor inhibited basal Δ^5-3β-hydroxysteroid dehydrogenase-isomerase activity in cultured Leydig cells from immature rats in a concentration- and time-dependent manner. Maximal inhibition was achieved with 5-10 ng/ml basic fibroblast growth factor following approximately 48 h of exposure. The inhibition of basal Δ^5-3β-hydroxysteroid dehydrogenase-isomerase activity was not altered by human chorionic gonadotropin; however, cycloheximide (0.5-2.0 μg/ml) partially reversed the effects of basic fibroblast growth factor in a dose-dependent manner. These studies suggest that locally-produced basic fibroblast growth factor may modulate Leydig cell testosterone formation by regulating Δ^5-3β-hydroxysteroid dehydrogenase-isomerase activity.

Basic FGF is a multifunctional peptide which stimulates the proliferation and modulates the differentiation of many mesoderm- and neuroectoderm-derived cells (1). Basic FGF also has angiogenic effects (2) and is widely distributed in many cell types (3). Among steroidogenic tissues bFGF has been identified in adrenocortical cells (4), ovarian granulosa cells (5) and testes (6).

In neonatal cultured rat testicular cells, bFGF was reported to inhibit LH-stimulated testosterone production after 24 h of exposure, and the effect was more pronounced at 48 and 72 h of exposure (7). However, in immature cultured porcine Leydig cells, FGF was reported to initially stimulate (24 h after exposure), then subsequently inhibit down to basal levels (48 h after exposure) hCG-stimulated testosterone production (8). We recently showed that bFGF at

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Abbreviations: Δ^5-3β-HSD, Δ^5-3β-hydroxysteroid dehydrogenase-isomerase; bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; F-12, Ham's F-12 nutrient mixture; HEPES, N-[2-hydroxy-ethyl] piperazine-n'-[2-ethanesulfonic acid]; hCG, human chorionic gonadotropin, ITLC, instant thin-layer chromatography sheets.

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lower concentrations (0.2-2 ng/ml) actually increased hCG-stimulated testosterone formation in cultured immature rat Leydig cells (due to an inhibition of 5α-reductase activity which reduced the level of testosterone metabolism), while at higher concentrations (5-25 ng/ml) bFGF actually inhibited hCG-stimulated testosterone formation (9). Because Δ^5-3β-HSD is an obligatory enzyme involved in the biosynthesis of testosterone, the present studies examined the possibility that this decline in testosterone production was due, in part, to an inhibition of this enzyme by bFGF.

MATERIALS AND METHODS

Materials and animals. Collagenase (Type 1), hCG, dimethyl sulfoxide, penicillin G, streptomycin and cycloheximide were from Sigma Chemical Co., St. Louis, MO. Basic FGF was from Amgen, Thousand Oaks, CA. ITLC sheets were from Gelman Sciences, Ann Arbor, MI. DMEM, F-12, HEPES buffer and $NaHCO_3$ were from Grand Island Biological Co., Grand Island, NY. Percoll was from Pharmacia Co., Piscataway, NJ. [7-^{3}H] Pregnenolone (23 Ci/mmol) and [4-^{14}C] progesterone (57 mCi/mmol) were from DuPont Co., Boston, MA. Unlabeled pregnenolone and progesterone were from Steraloids, Wilton, NH. Organic solvents were from Fisher Scientific Co., Atlanta, GA.

Sprague-Dawley rats were purchased at the time of weaning from Zivic-Miller Lab., Zelienople, PA. At 25 days of age, they were rendered unconscious in a saturated CO_2 chamber, then killed by decapitation between 0800 and 0900h. Testes were excised, decapsulated and dispersed with collagenase to obtain interstitial cells (10).

Isolation and culture of immature Leydig cells. Interstitial cells were layered over a 20 ml continuous 15-60% Percoll gradient and centrifuged at 3300xg for 15 min to isolate Leydig cells (11). The third band of cells containing the majority of Leydig cells was isolated and washed thrice in culture medium consisting of DMEM/F-12 (1:1 mixture) containing 1.2 g/l $NaHCO_3$, 15 mM HEPES, 100 U/ml penicillin G and 100 μg/ml streptomycin. Cells were resuspended in the same culture medium to give a final concentration of approximately 10^5 cells/ml, and 1 ml of cells was plated in each 16 mm diameter well of a 24-well Costar culture dish. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2 (11). Media were changed 24 h after plating and treatment initiated.

Δ^5-3β-Hydroxysteroid dehydrogenase-isomerase assay on cultured cells. Δ^5-3β-HSD activity in cultured cells generally was measured 48 h after start of treatment or at designated intervals for the time study. To measure Δ^5-3β-HSD activity of cultured cells, media were changed twice and cells preincubated for 30 min to removed accumulated steroids (12). Cells were washed two additional times with fresh media, and the reaction was initiated by the addition of 1 ml of medium containing [^{3}H]pregnenolone (10 μM, 0.5 μCi), 10 μM spironolactone (to inhibit 17α-hydroxylation of pregnenolone and the product progesterone) (13) and dimethyl sulfoxide (0.3% final concentration). The reaction period was 30 min at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The reaction was terminated by the addition of 0.1 ml of 1 N NaOH, and 100 μg each of pregnenolone and [^{14}C] progesterone (~4000 cpm) were added as carriers and for recovery estimates. Samples were extracted with 5 vol of diethyl ether, and the residues of ether extracts were chromatographed by ITLC using a solvent system of chloroform: methanol (99.5:0.5, vol:vol). Progesterone was localized using iodine vapors and counted in Omnifluor-toluene.

Statistical analysis. Each study is representative of at least three independent experiments. Comparison of several treatment groups was performed using analysis

of variance. Treatment differences were evaluated using Duncan's new multiple range test. Comparison of two treatment groups (time study) was performed by Student's *t* test. A p value of <0.05 was considered statistically significant.

RESULTS

Effect of increasing bFGF concentrations on basal Δ^5-3β-HSD activity in cultured immature Leydig cells. Basal Δ^5-3β-HSD activity in immature Leydig cells on day 3 of culture was 0.406 $\pm$ 0.070 nmol progesterone/30 min/10^5 cells (Fig. 1). In the presence of 1 ng/ml bFGF enzyme activity declined to 0.220 $\pm$ 0.025 nmol ($p<0.01$), and further declined to 0.055 $\pm$ 0.008 nmol ($p<0.01$) in the presence of 5 ng/ml bFGF. Δ^5-3β-HSD activity remained unchanged at the next two higher bFGF concentrations (5 and 10 ng/ml), but then increased slightly (although not statistically significant) at 100 and 200 ng/ml bFGF. We observed this tendency to gradually increase at higher bFGF concentrations in three separate experiments.

Effect of time of exposure to 10 ng/ml bFGF on Δ^5-3β-HSD activity of cultured immature Leydig cells. Following 6 h of exposure to 10 ng/ml bFGF Δ^5-3β-HSD activity was unchanged when compared to untreated cells (Fig. 2). However,

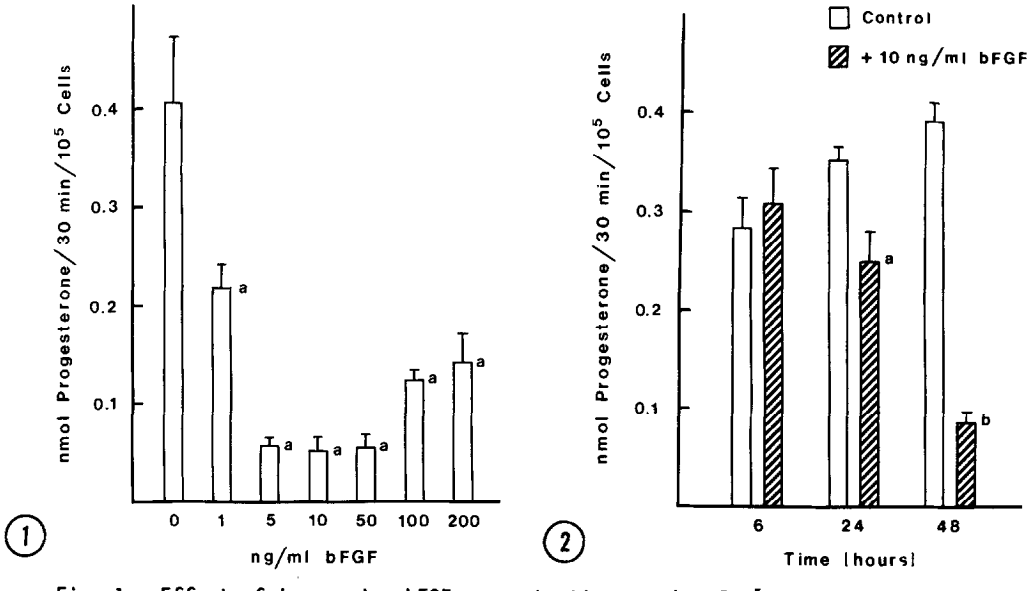

Fig. 1. Effect of increasing bFGF concentrations on basal Δ^5-3β-hydroxysteroid dehydrogenase-isomerase activity in cultured immature Leydig cells. Cells were exposed to bFGF for 48 h. Each treatment group represents the mean $\pm$ SEM of four separate determinations. a: $p<0.01$ when compared to untreated control.

Fig. 2. Effect of exposure time to bFGF on Δ^5-3β-hydroxysteroid dehydrogenase-isomerase activity in cultured immature Leydig cells. Concentration of bFGF was 10 ng/ml. Each treatment group represents the mean $\pm$ SEM of four separate determinations.
a: $p<0.05$ when compared to control of same time period.
b: $p<0.01$ when compared to control of same time period.

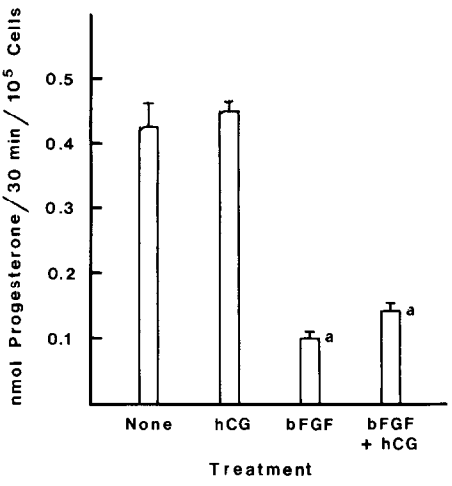

Fig. 3. Effect of hCG on inhibition of Δ^5-3β-hydroxysteriod dehydrogenase-isomerase activity by bFGF in cultured immature Leydig cells. Concentration of bFGF was 10 ng/ml. Each treatment group represents the mean $\pm$ SEM of four separate determinations. Treatment period was 48 h.
a: $p<0.01$ when compared with untreated control.

following 24 h of exposure, Δ^5-3β-HSD activity of treated cells declined to 71% of control ($p<0.05$), and following 48 h of exposure activity of treated cells further declined to 22% of control ($p<0.01$). We examined the inhibitive effect of bFGF after 96 and 144 h of exposure, and the pattern was similar to that observed after 48 h (data not shown).

Effect of hCG on bFGF inhibition of Δ^5-3β-HSD activity in cultured immature Leydig cells. Because LH/hCG is the primary hormone regulating testosterone formation by Leydig cells, we examined whether hCG could alter or reverse the inhibitive effects of bFGF on Δ^5-3β-HSD activity of cultured immature Leydig cells. Treatment with 10 mIU/ml hCG alone for 48 h had no effect on Δ^5-3β-HSD activity of cultured immature Leydig cells, and hCG did not reverse the inhibitive effects of 10 ng/ml bFGF (Fig. 3).

Effect of increasing cycloheximide concentrations on inhibitive effects of bFGF on Δ^5-3β-HSD activity of cultured immature Leydig cells. To examine the possibility that the inhibition of Δ^5-3β-HSD activity of cultured immature Leydig cells was due to the stimulation of protein synthesis by bFGF, cells treated with bFGF were exposed to increasing cycloheximide concentrations (0.5-2.0 μg/ml) for 48 h (Fig. 4). The inhibitive effects of 10 ng/ml bFGF were progressively reversed ($p<0.01$) by increasing cycloheximide concentrations. Although the highest concentration of cycloheximide did not return Δ^5-3β-HSD activity to untreated control levels, activity was more than 2.5-fold higher than cells treated with bFGF alone. We examined higher cycloheximide concentrations (5-20 μg/ml) but observed some toxicity when exposure times were greater than 24 h (data not shown).

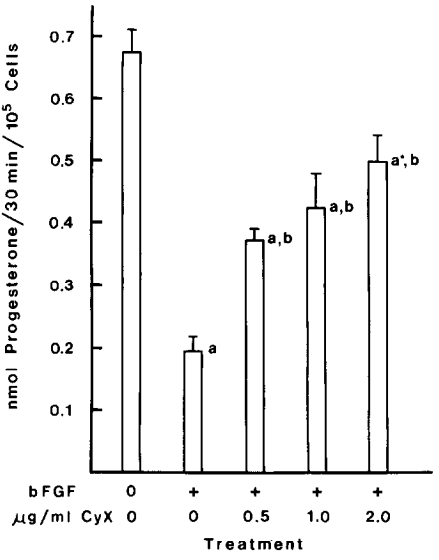

Fig. 4. Effect of increasing concentrations of cycloheximide on inhibition of Δ^5-3β-hydroxysteriod dehydrogenase-isomerase activity by bFGF in cultured immature Leydig cells. Cells were exposed to 10 ng/ml bFGF alone or with CyX for 48 h. Each treatment group represents the mean ± SEM of four separate determinations.
a: $p<0.01$ when compared to untreated control.
a*: $p<0.05$ when compared to untreated control.
b: $p<0.01$ when compared to cells treated with bFGF alone.

DISCUSSION

The present studies demonstrate that bFGF is a potent inhibitor of Δ^5-3β-HSD activity in cultured immature Leydig cells, thus raising the possibility that locally-produced bFGF may reduce testosterone formation in Leydig cells by inhibiting this enzyme.

Previous studies have shown that FGF inhibits LH/hCG-stimulated testosterone formation in neonatal (7) and immature rat Leydig cells (9). Studies using neonatal testicular cells demonstrated that the inhibition of LH-stimulated testosterone formation by bFGF was accompanied by a 12-fold increase in progesterone levels, suggesting that the inhibitory effect was localized at a step distal to progesterone formation (most likely 17α-hydroxylase activity) (7). However, the present studies clearly reveal that in cultured immature Leydig cells bFGF inhibits Δ^5-3β-HSD activity, the enzyme which directly converts pregnenolone to progesterone. This may suggest that fetal/neonatal Leydig cells differ from immature Leydig cells with respect to sensitivity to bFGF.

We recently showed that bFGF inhibited LH- and 8-bromo-cAMP-stimulated 5α-reductase activity in cultured immature Leydig cells but had no effect on basal enzyme activity (9). Because bFGF inhibited basal Δ^5-3β-HSD activity in cultured immature Leydig cells in the present study, this would suggest that mechanisms regulating Δ^5-3β-HSD and 5α-reductase activities differ in these cells. Under

the present conditions hCG had no effect on Δ^5-3β-HSD activity of cultured immature Leydig cells and did not alter the effects of bFGF; however, cycloheximide was able to partially reverse the effects of bFGF. This suggests that the action of bFGF on Δ^5-3β-HSD activity is mediated by a protein(s) which is stimulated by bFGF. Presently we do not have evidence whether this effect is mediated directly on Leydig cells or through some other cell type present in interstitial tissue.

A recent study identified an FGF-like factor in cultured Sertoli cells from immature rats. Thus, results of the present studies raise the possibility that locally produced bFGF may modulate testosterone formation by regulating Δ^5-3β-HSD activity.

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