Catecholamine-induced stimulation of testosterone production by Leydig cells from fetal mouse testis

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Summary. In contrast to the strong stimulation of testosterone production by hCG, L-isoproterenol had little effect on freshly isolated Leydig cells from 18-day-old mouse fetuses. However, the ability of fetal Leydig cells to respond to L-isoproterenol exposure increased during culture (0–24 h). The response of the cultured cells to L-isoproterenol was dose-dependent with an ED_{50} at 2×10^{-7} M. Adrenaline and noradrenaline at a concentration of 10^{-5} M also increased testosterone production by cultured fetal Leydig cells. DL-Propranolol, a β -antagonist, inhibited L-isoproterenol-stimulated testosterone production in a dose-dependent manner, while phentolamine, an α -adrenergic antagonist, had no effect. These results suggest that catecholamines may play an essential role in the control of testicular steroidogenesis during fetal development.

Introduction

A direct involvement of catecholamines in adult testicular function has been suggested by results from experiments with isolated interstitial cells. Isoproterenol, adrenaline and noradrenaline have been shown to stimulate both cyclic AMP accumulation and testosterone production (Cooke *et al.*, 1982; Moger *et al.*, 1982) by interacting with specific receptors located on the Leydig cell surface (Poyet & Labrie, 1983; Anakwe *et al.*, 1985). Such a stimulatory effect was, however, only observed in cultured Leydig cells and not in freshly isolated cells or whole decapsulated testis. During fetal development, catecholamines are present in high concentrations in the amniotic fluid and fetal circulation (Zuspan *et al.*, 1974; Ben-Jonathan & Maxson, 1978). In addition, maximal levels of biogenic amines have been detected in the testis of neonatal rats of 1-day of age as compared with prepubertal and adult animals (Zieher *et al.*, 1971). We have reported that isoproterenol, a β -adrenergic agonist, is able to stimulate testosterone production by mouse fetal Leydig cells in primary culture (Pointis & Latreille, 1986), and that fetal Leydig cell steroidogenesis could be modulated by catecholamines. In the present study the dose-response to isoproterenol and the acquisition of catecholamine responsiveness during the culture period were determined. In addition we examined the effect of adrenaline, noradrenaline, propranolol (a β -adrenergic antagonist) and phentolamine (an α -adrenergic antagonist) on testosterone production by fetal Leydig cells.

Materials and Methods

Chemicals. hCG (2nd IS for chorionic gonadotrophin for bioassay, 2180 i.u./mg) was provided by the National Institute for Biological Standards and Control (London, U.K.). L-Isoproterenol, L-adrenaline, L-noradrenaline, DL-propranolol, 3-isobutyl-1-methylxanthine (MIX) and ascorbic acid were commercial preparations from Sigma Chemical Co. (St Louis, MO, U.S.A.). Phentolamine was a gift from Ciba-Geigy A.G. (Basel, Switzerland). N-2 hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes) and bovine serum albumin (BSA) were purchased from Eurobio (Paris, France) and ICN Pharmaceuticals Inc. (Cleveland, OH, U.S.A.), respectively. All other chemicals were from Difco Laboratories (Detroit, MI, U.S.A.).

Leydig cell preparation. Leydig cells were isolated from 18-day-old fetal Swiss mice by mechanical dissection and collagenase digestion as previously reported (Pointis *et al.*, 1984). Cell viability was tested by Trypan blue dye exclusion and Leydig cells were identified by staining for 3β -hydroxysteroid dehydrogenase (3β -HSD) using a procedure previously described (Pointis & Latreille, 1985). Leydig cells were cultured in Medium 199 supplemented with 15 mM-Hepes, 0.1% BSA, 0.5 g glucose/l, 100 u penicillin/ml and 100 μ g streptomycin/ml in Falcon tissue-culture dishes (diameter 15 mm) at a density of 1.5×10^4 3β -HSD-positive cells/dish. At different times after the start of the culture (0, 6, 12, 24 h) the culture medium was replaced with fresh medium modified to contain 0.1 mM-MIX, a phosphodiesterase inhibitor which increases endogenous levels of cAMP, and 0.1 mM-ascorbic acid to reduce breakdown of catecholamines. L-Isoproterenol, L-adrenaline, L-noradrenaline, DL-propranolol and phentolamine were dissolved in this medium and added to the culture dishes as 10 μ l at the concentration required. The hCG was used at a concentration which gave maximal testosterone response as previously determined (Pointis & Latreille, 1985). The cells were subsequently incubated for 3 h at 37°C in an atmosphere of 95% air, 5% CO₂. At the end of the incubation, media were removed by aspiration, centrifuged at 3000 g for 20 min and stored at -30°C for measurement of testosterone.

Testosterone assay. Testosterone concentrations in the culture media were assayed after appropriate dilutions by a specific radioimmunoassay as previously described (Pointis *et al.*, 1984) and expressed as ng/10⁴ 3β -HSD-positive cells. The specific antibody to testosterone was purchased from Institut Pasteur Production, Paris, and [1,2,6,7-³H]testosterone (sp. act. 92 Ci/mmol) was from the Radiochemical Centre, Amersham, Bucks, U.K. The sensitivity of the assay was 7 pg/sample. The inter- and intra-assay coefficients of variation for the assay were <8%. Cross-reaction with 5 α -dihydrotestosterone was 36% but was <2% with other steroid hormones. Since the testosterone values were comparable with or without celite chromatography (Pointis & Mahoudeau, 1974), this step was omitted.

Statistical analysis. Results were analysed by analysis of variance (ANOVA), and the significance of differences between treatment group means was determined by Duncan's new multiple range test.

Results

Catecholamine stimulation of testosterone production

In agreement with our previous findings (Pointis & Latreille, 1986), isoproterenol, a non-selective β -agonist, stimulated testosterone production when added to fetal Leydig cells on Day 2 of culture at concentrations ranging from 10^{-9} to 10^{-5} M (Fig. 1). A dose-response curve for isoproterenol stimulation was obtained with half-maximum stimulation value estimated at about 2×10^{-7} M. Testosterone production reached maximal values in the presence of 10^{-5} M-isoproterenol. Adrenaline and noradrenaline, at a concentration of 10^{-5} M, also had a stimulatory effect on testosterone production. The mean $\pm$ s.e.m. ($n = 3$) levels of testosterone in the presence of these agents were respectively 3.40 ± 0.25 and 2.70 ± 0.08 ng/10⁴ 3β -HSD-positive cells. These levels were significantly higher than the control values of 1.33 ± 0.15 ng/10⁴ 3β -HSD-positive cells ($P < 0.01$). Figure 2 shows that treatment of control cells with a maximal stimulating dose of hCG (100 mi.u./ml) brought about a substantial increase in testosterone accumulation ($P < 0.001$). No additive effect of isoproterenol and hCG could be detected for any concentrations of the β -adrenergic agent tested (10^{-9} to 10^{-5} M).

Acquisition of catecholamine responsiveness during culture period

The response of fetal Leydig cells to isoproterenol, characterized as percentage of basal testosterone production (stimulated - basal/basal) $\times$ 100) to eliminate differences in basal production on different days of the experiment, appeared to be time-dependent over the period studied: values were 23.7 ± 5.3 (4 replicates), 53 ± 2 (2), 72.7 ± 3.7 (2), 87.7 ± 12.5 (4) at 0, 6, 12 and 24 h respectively. Statistical analysis of the data revealed a significant effect of the time in culture on the acute response to isoproterenol ($F(3,8) = 10.29$, $P < 0.01$). Freshly isolated fetal Leydig cells responded to isoproterenol with only a slight increase in testosterone production while a maximal dose of hCG (100 mi.u./ml) induced a 6-fold increase (control: 1.00 ± 0.05 ng testosterone/10⁴ 3β -HSD-positive cells; hCG-stimulated cells: 6.05 ± 0.15 ng testosterone/10⁴ 3β -HSD-positive cells). The stimulatory effect of isoproterenol on testosterone production increased gradually thereafter with a maximal response observed after 24 h of culture.

Fig. 1. Isoproterenol stimulation of testosterone production by fetal mouse Leydig cells in primary culture. Testosterone was measured in the medium after 3 h incubation in the presence of increasing concentrations of isoproterenol on Day 2 of culture. Control refers to testosterone production by unstimulated cells. Vertical lines indicate the s.e.m. of triplicate cultures. Results are representative of 2 experiments.

Fig. 2. Effect of concomitant addition of hCG and isoproterenol to the culture medium of fetal mouse Leydig cells. On Day 2 of culture, fetal Leydig cells were incubated for 3 h in the presence of increasing doses of isoproterenol plus or minus a maximal dose of hCG (100 mi.u./ml). Control refers to testosterone production by unstimulated cells. Results are the mean $\pm$ s.e.m. of triplicate cultures and are representative of 3 experiments.

Fig. 3. Effect of propranolol on isoproterenol-stimulated testosterone production by fetal mouse Leydig cells in primary culture. Testosterone was measured in the medium after 3 h incubation with isoproterenol (10^{-5} M) in the presence of increasing concentrations of propranolol on Day 2 of culture. Results are the mean $\pm$ s.e.m. of triplicate cultures and are representative of 2 experiments.

Specificity of the catecholamine response

The specificity of the β -adrenergic response was further investigated with two antagonists. As shown in Fig. 3, the stimulatory effect of isoproterenol on testosterone production was prevented in a dose-dependent manner by concomitant exposure of fetal Leydig cells to propranolol, a non-selective β -adrenergic antagonist. Half-maximum inhibition was observed for 4.5×10^{-7} M and a total abolition of isoproterenol-stimulated testosterone production with 10^{-4} M-propranolol. Due to the limited fetal material, the effect of phentolamine, a competitive α -receptor antagonist, on the isoproterenol-stimulated testosterone production was investigated in a subsequent experiment. There were no significant differences in testosterone production between isoproterenol-stimulated cells with or without 10^{-4} M-phentolamine. The mean ($\pm$ s.e.m.) testosterone values (ng/10⁴ 3 β -HSD-positive cells) of quadruplicate cultures were 1.61 ± 0.06 in control, 2.66 ± 0.23 in the presence of isoproterenol and 2.17 ± 0.17 in the presence of isoproterenol and phentolamine.

Discussion

The present study shows clearly that catecholamines are able to stimulate the production of testosterone by fetal Leydig cells derived from testes of 18-day-old mouse fetuses. The ability of propranolol, a β -adrenergic antagonist, to block in a dose-dependent manner the stimulatory effect of isoproterenol and the lack of effect of the α -antagonist, phentolamine, suggest that this effect is probably mediated via β -adrenergic receptors. These data confirm and extend reports (Anakwe & Moger, 1984; Pointis & Latreille, 1986) that the fetal testis, like adult gonadal tissue (Cooke *et al.*, 1982; Moger *et al.*, 1982), can be directly affected by exposure to catecholamines.

It is unlikely that the response of fetal Leydig cells to catecholamines is due to an indirect effect of these agents on contaminant cells via their cAMP production. On the one hand the most likely

contaminant cells (erythrocytes and fibroblasts) are unable to produce measurable amounts of cAMP in response to catecholamines (Jordan *et al.*, 1978). On the other hand we have previously reported that large amounts of cAMP are necessary to induce a significant increase in testosterone production in such a preparation of fetal Leydig cells (Pointis & Latreille, 1985). Moreover, the sensitivity of whole decapsulated fetal testis to isoproterenol was not higher than that observed in the present study with purified fetal Leydig cells, suggesting a lack of significant effect of the other cell type (Anakwe & Moger, 1984). Lastly, the fact that, after 24 h in culture, maximum concentrations of catecholamines stimulate testosterone production with a potency slightly lower than that elicited with maximal hCG stimulation, indirectly suggests that both hormones most probably affect the same cell type. Taken together, these data therefore suggest that β -adrenergic stimulation of testosterone production is likely to be confined to Leydig cells.

The mechanism of development of catecholamine responsiveness during the culture period is presently unclear. Such an acquisition of the response to β -adrenergic agonist has been reported for different cell types such as hepatocytes (Nakamura *et al.*, 1983), granulosa cells (Kliachko & Zor, 1981) and Sertoli cells (Kierszenbaum *et al.*, 1985). As shown in the current study, β -agonists did not affect or only slightly increased testosterone production in fresh interstitial cells derived from adult testis (Cooke *et al.*, 1982; Moger *et al.*, 1982). The fetus responds to direct stimuli such as asphyxia, hypoglycaemia or cold exposure with noradrenaline secretion (Comline *et al.*, 1965; Eales & Small, 1980). It is therefore possible that the stressful conditions for obtaining fetal material induced increased noradrenaline accumulation in the fetal bloodstream and subsequently results in a β -adrenergic desensitization. Such an hypothesis was, however, not confirmed with adult Leydig cells (Cooke *et al.*, 1982; Moger & Murphy, 1983). Whether the appearance of a β -adrenergic response during culture of fetal Leydig cells is due to synthesis of a new β -adrenergic receptor (Nakamura *et al.*, 1983), altered differentiation (Moger & Murphy, 1983) or other processes as suggested for adult cells is presently not clear.

High levels of catecholamines, from a fetal origin, have been measured in amniotic fluid and the fetal circulation (Ben-Jonathan & Maxson, 1978). It is therefore tempting to speculate that *in vivo* these agents reach the fetal testis and exert a local regulation within the fetal testis. On the other hand, the presence of adrenergic axons innervating testicular interstitial tissue was reported for the adult (Goujard, 1954; Hodson, 1970; Lawrence & Burden, 1976; Frankel & Ryan, 1981). Such a direct involvement of the autonomic nervous system is likely to occur during fetal development since neural pathways innervating the fetal urogenital complex have been characterized (Brauer *et al.*, 1985).

In conclusion, although it is well established that LH is the main gonadotrophic hormone which controls testosterone synthesis in the fetal testis, the present observations that catecholamines stimulate testosterone production *in vitro* by fetal Leydig cells suggest that these β -adrenergic agents are also involved in the regulation of the fetal testicular function.

We thank Dr L. Cedard and Dr B. Rao for helpful advice and criticism of the text and Mrs M. Verger for typing the manuscript.

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Received 27 October 1986