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INSULIN-LIKE GROWTH FACTOR I (IGF I) INDUCES CORTISOL PRODUCTION IN BOVINE ADRENOCORTICAL CELLS IN PRIMARY CULTURE

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Summary—The effects of a physiological dose of IGF I (40 ng/ml $\approx 5 \times 10^{-9}$ M) on steroidogenesis were studied in bovine adrenal fasciculata cells cultured in serum-free McCoy's medium. They were compared with those of a single dose of ACTH (0.25 ng/ml $\approx 10^{-10}$ M) at approximately the concentration inducing half-maximal stimulation. With IGF I, steroidogenesis commenced after 48 h culture and progressively increased throughout the 96-h test period. Expressed as stimulated level/control level ratios, glucocorticoid (cortisol + corticosterone) responses to IGF I after 4 days' culture (2.41 ± 0.20 (SEM) $n = 9$) were similar to those obtained with ACTH (2.59 ± 0.18, $n = 9$). A combination of the two peptides had a synergistic effect (5.95 ± 0.79, $n = 5$).

The cortisol/corticosterone ratio increased in the presence of IGF I from 1 ± 0.19 to 1.76 ± 0.45 ($n = 7$, $P < 0.02$), although less so than in the presence of ACTH (5.50 ± 0.98). Moreover, cortisol production was accompanied by adrostenedione production (2.36 ng/10^6 cells, $n = 3$) similar to that induced by ACTH (2.10 ng/10^6 cells, $n = 3$). These findings together suggest stimulation of 17α-hydroxylase activity. Cell multiplication was unaffected by IGF I. [^{3}H]Thymidine incorporation into DNA reached only 193% ± 17 (SEM) ($n = 4$) of control levels, whereas with ACTH it dropped to 60% ± 5.

Our findings show that IGF I alone has no mitogenic effect on adrenocortical cells *in vitro*, but that it is capable of inducing differentiated steroidogenesis.

INTRODUCTION

The roles of growth factors in cell maturation/differentiation have been studied extensively over the past few years. Various tissues have been used, with gonads and particularly granulosa cells taking priority in attempts to determine whether or not they produce insulin-like growth factors (IGFs) and what role the IGFs may have in the differentiation of ovarian function [1]. The effects of the IGFs appear to come into play very early, since IGF I has been reported to induce cytochrome P-450$_{scc}$ and adrenodoxin synthesis in ovarian cells [2]. Little work has been devoted to the adrenal cortex. Morera *et al.* [3] have indicated that IGF I potentiates the effects of ACTH and angiotensin II on bovine adrenal steroidogenesis and IGF I receptors have recently been identified on bovine adrenal fasciculata cells [4]. Increases have been seen in the hydroxylase activities governing cortisol biosynthesis [4] and, in addition, we have noted that human adrenal cells taken from a Conn's adenoma are capable of secreting IGF I and IGF II [5].

In this study we set out to determine whether or not IGF I alone was capable of inducing steroidogenesis in bovine adrenocortical cells in primary culture and to compare the levels of cortisol production obtained with IGF I with those observed under the influence of the major regulator of adrenal hydroxylase biosynthesis and stimulation, ACTH.

EXPERIMENTAL

Adrenals from 2–3-year-old steers were collected at the abattoir and transplanted to the laboratory in 0.9% NaCl on ice. Isolated cells were obtained by a technique modified according to Gospodarowicz *et al.*[8] from that employed to isolate guinea-pig adrenocortical cells [6, 7].

The following protocol was adopted: adrenals were thoroughly washed in sterile 0.9% NaCl, fat was removed and dissection done with a microtome. The capsule and medulla were discarded. The slices containing the cortex were washed with Hepes-buffered (pH 7.4) McCoy's medium containing 100 U/ml penicillin, 50 μg/ml streptomycin and 20 μg/ml gentamycin. They were then digested with an enzyme solution in the same medium containing collagenase (Boehringer, 0.5 mg/ml), deoxyribonuclease (Sigma, 50 μg/ml) and 5% foetal calf serum (Gibco). Digestion for 1 h at 37°C with mechanical stirring was repeated four times. The supernatant of the first digestion was discarded and the subsequent

three supernatants filtered over sterile surgical gauze and then centrifuged for 15 min at 100 *g*. The cell pellets were washed in the same medium and recentrifuged. In order to obtain homogenous fasciculata cells, suspensions of 100 million cells/2 ml medium were loaded onto a discontinuous Percoll gradient (Pharmacia) and the cells separated by centrifugation for 30 min at 1250 *g* [9]. The fasciculata cells thus free of debris and red blood cells were seeded either at $3–4 \times 10^5$ cells in 24-well plates in 1 ml McCoy's medium with antibiotics and 10% foetal calf serum, or at $1–2 \times 10^6$ cells in 6-well plates in 4 ml of the same medium. They were incubated at 37°C for 48–72 h in a moist atmosphere containing 5% CO_2. Thereafter, the serum-containing medium was removed and the cells washed three times in 0.9% NaCl. After being re-incubated in serum-free McCoy's medium (1 or 4 ml), the cells were treated with ACTH 1-24 (Syncathen Ciba, 0.25 ng/ml), IGF I (Amgen, 40 ng/ml) and/or insulin (Novo, 27 U/mg, 300 ng/ml). The insulin concentration used greatly exceeded physiological levels. In all experiments, except those done to investigate steroid production as a function of time, media were collected after 4 days of culture.

Total glucocorticoids (cortisol + corticosterone) were assayed by competitive binding with human transcortin [10]. Cortisol and corticosterone were measured specifically after chromatographic separation on Sephadex LH 20 (Pharmacia) in methanol–dichloromethane (2/98, v/v). Androstenedione, separated on Sephadex LH 20 in heptane–chloroform (50/50, v/v), was measured by RIA [11].

Incorporation of tritiated thymidine (Amersham) into DNA was measured after incubations with 1 μCi/ml during the final 3 days of culture. At the end of this period, the medium was removed and the cells were washed three times in 5% trichloroacetic acid and solubilized in 0.5 N NaOH. Radioactivity was counted in an LKB counter after addition of Ready Protein cocktail (Beckman).

Cells were counted in a Malassez hematocytometer following detachment with 0.25% trypsin in 0.02% EDTA (Eurobio) for 15 min at 37°C and neutralization of enzyme action by addition of 10% foetal calf serum in McCoy's medium.

RESULTS

Choice of ACTH concentration

In bovine adrenocortical cells cultured in serum-free McCoy's medium with varying concentrations of ACTH for 24 or 96 h, stimulation of corticosteroidogenesis was dose-dependent. However, as shown in Fig. 1, the dose–response curves varied widely from one experiment to another. An average concentration was therefore chosen in the region yielding half-maximal stimulation (0.25 ng/ml $\approx 10^{-10}$ M) to allow for a response to ACTH and, at the same time, any further response if the combination of IGF I and ACTH were to provoke increased steroidogenesis.

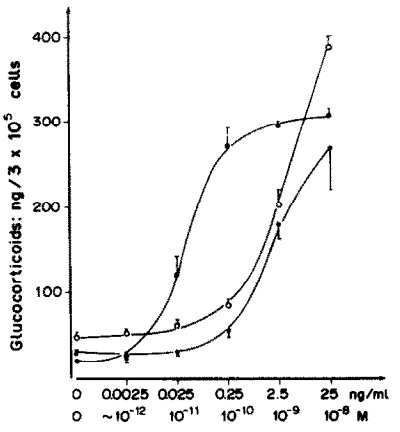

Fig. 1. Effects of ACTH on glucocorticoid secretion by bovine adrenocortical cells. Cells were seeded at 3×10^5 in 24-well plates in 1 ml McCoy's medium with 10% foetal calf serum and incubated at 37°C with 5% CO_2 for 48 h. Thereafter, the serum-containing medium was removed and the cells were washed three times with 0.9% NaCl and re-incubated with 1 ml serum-free medium in the absence and in the presence of varying concentrations of ACTH (from 0.0025 ng/ml $\approx 10^{-12}$ M to 25 ng/ml $\approx 10^{-8}$ M) for 24 or 96 h. Each experimental point represents the mean of quadruplicate incubations (±SEM). The curves represent data from three separate cell culture experiments.

Glucocorticoid production: time-course studies

This concentration of ACTH (0.25 ng/ml), added in a single dose when the cells were transferred to serum-free McCoy's medium, induced glucocorticoid production which reached a plateau after 4 h incubation (128 ± 4 (SEM) ng/3×10^5 cells/ml). A physiological dose of IGF I (40 ng/ml $\approx 5 \times 10^{-9}$ M) increased glucocorticoid production within 48 h from

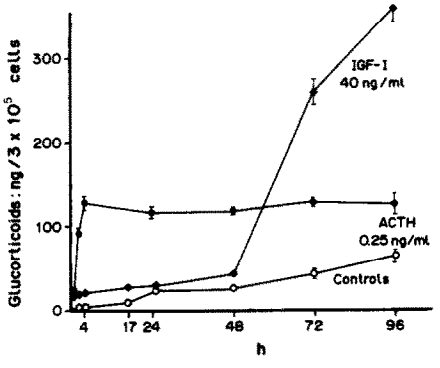

Fig. 2. Time-course studies of steroid production (cortisol + corticosterone) in the presence of a single dose of either 0.25 ng/ml ACTH or 40 ng/ml IGF I. Cells were incubated in 1 ml serum-free medium in the absence ○-----○ and in the presence of ACTH ●-----● of IGF I ◆-----◆ for the periods of time indicated. In the experiment shown, the final glucocorticoid level obtained with IGF I exceeded that obtained with ACTH. Each experimental point is the mean of triplicates (±SEM) and represents cumulative production from 0 h. For the points where bars are missing, the 3 values were nearly identical.

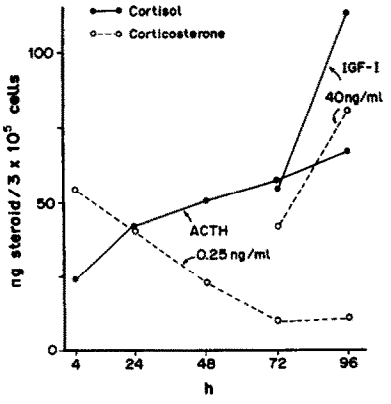

Fig. 3. Changes in cortisol and corticosterone levels in the experiment illustrated in Fig. 2. Measurements were done after chromatographic separation of Sephadex LH 20 (see Materials and Methods). Because of the amount of medium available and steroids produced, the levels reached in the presence of IGF I were measurable only after 72 h culture.

$24 + 0.3$ to 42 ± 1.4 ng/3 $\times 10^5$ cells/ml. There were considerable further increases at 72 (261 ± 15 ng) and 96 (358 ± 17 ng) h (Fig. 2).

From the specific assays of cortisol and corticosterone it could be seen that cortisol production increased progressively from 4 to 96 h of culture under the influence of ACTH, whereas corticosterone production dropped significantly (Fig. 3). Under the influence of IGF I, the production of both steroids increased.

Glucocorticoid production: levels and cortisol/corticosterone ratio

Using the 4-day culture period in serum-free medium, a series of 9 further experiments was done to test ACTH, IGF I, ACTH + IGF I and insulin in parallel. Cells were initially seeded in either 24- or 6-well plates at the densities indicated in Materials and Methods. The results are shown in Table 1 and are given per 10^6 cells throughout. The mean glucocorticoid responses obtained are expressed as stimulated level/control level ratios. The response obtained with 40 ng/ml IGF I (2.41 ± 0.20 (SEM), $n = 9$) was very close to that obtained with 0.25 ng/ml ACTH (2.59 ± 0.18, $n = 9$). The two peptides together had a synergistic effect, raising glucocorticoid production to 5.95 ± 0.79 ($n = 5$). However, supra-physiological doses of insulin (300 ng/ml) induced only slight stimulation.

Analysis of the relative amounts of cortisol and corticosterone produced in this series of experiments (Fig. 4), showed a distinct increase in the cortisol/corticosterone ratio (F/B) from 1 ± 0.19 to 1.76 ± 0.45 (SEM) ($n = 7$, $P < 0.02$, Wilcoxon's t-test) under the influence of IGF I. This was nevertheless not as large as the increase seen either under the influence of ACTH (5.5 ± 0.93, $n = 8$) or with the combination of IGF I and ACTH (10.32 ± 3.56, $n = 4$). With insulin, the ratio remained unchanged.

Androstenedione was measured in 3 experiments. IGF I-induced production was similar to that induced by ACTH (Table 2). The IGF I-ACTH combination had a potentiating effect.

Tritiated thymidine incorporation into DNA and cell multiplication

The amount of 40 ng/ml IGF I induced increased [^{3}H]thymidine incorporation into DNA, which, however, did not exceed $193\% \pm 17$ ($n = 4$) of control levels. ACTH inhibited incorporation to $60\% \pm 5$. There was no difference in cell count between controls and those cultured in the presence of IGF I, ACTH or insulin (Table 3).

DISCUSSION

The effects of a physiological concentration of IGF I ($\approx 5 \times 10^{-9}$ M) have been studied on steroidogenesis in bovine adrenal fasciculata cells. These have been compared with the effects of a concentration of ACTH provoking approximately half-maximal stimulation. This ACTH concentration ($\approx 10^{-10}$ M) was one which fell between that reported for adrenocortical cells cultured in serum-supplemented

Table 1. Levels of glucocorticoid production reached after 4 days culture in serum-free McCoy's medium in the presence of a single dose of ACTH (0.25 ng/ml), IGF I (40 ng/ml), ACTH + IGF I or insulin (300 ng/ml)

Controls	ACTH (0.25 ng/ml)	IGF I (40 ng/ml)	ACTH + IGF I (0.25 + 40 ng/ml)	Insulin (300 ng/ml)
528	1620 (3.07)	987 (1.87)		
256	576 (2.25)	872 (3.41)		586 (2.29)
344	846 (2.46)	982 (2.85)		608 (1.77)
163	518 (3.18)	443 (2.72)		183 (1.12)
212	636 (3.00)	568 (2.68)	1280 (6.04)	440 (2.08)
192	328 (1.71)	268 (1.40)	1060 (5.52)	228 (1.19)
332	1010 (3.04)	744 (2.24)	2920 (8.80)	
446	1200 (2.69)	1140 (2.56)	1780 (3.99)	
250	490 (1.96)	480 (1.92)	1345 (5.38)	
Mean	(2.59)	(2.41)	(5.95)	(1.69)
SD	0.53	0.61	1.77	0.52
SE	0.18	0.20	0.79	0.23

Seeding density was 3–4 $\times 10^5$ cells in 24-well plates or 1–2 $\times 10^6$ cells in 6-well plates in 1 or 4 ml serum-free McCoy's medium. Results are expressed in ng glucocorticoid/10^6 cells. Stimulated level/control level ratios are shown in brackets. Mean, SD and SE refer to the ratios.

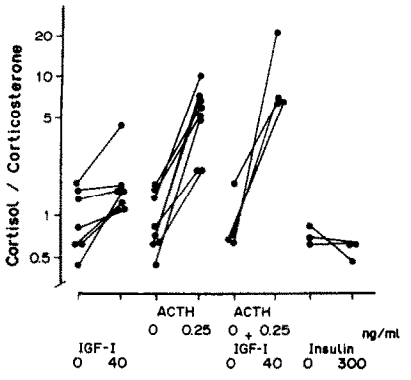

Fig. 4. Changes in cortisol/corticosterone ratios under the influence of IGF I, ACTH, ACTH + IGF I and insulin.

medium (10^{-9} M) [12] and that reported with isolated cell suspensions maintained in short-term incubation (10^{-11} M) [13].

With the addition of a single dose of ACTH at the time of the change-over to serum-free medium, maximal steroidogenesis was attained after 4 h culture and thereafter remained stable throughout the 4-day period studied. This rapid stabilization of cell response may be attributable to the single and relatively small dose of ACTH added. However, it may also be attributable to the fact that the medium was not renewed over the 4 days culture, thus causing inhibition of steroidogenesis by the corticosteroids produced and remaining in the medium [6, 14]. With a daily change of medium and addition of ACTH, the cells retained their steroidogenic capacity, but 24-h production gradually decreased after the first 24 h (unpublished data), a pattern which is well known for adrenocortical cells cultured in serum-containing medium [12, 15, 16]. With IGF I, daily renewal of the medium and addition of peptide did not yield significant corticosteroid production within 24 h and for this reason cumulative steroid production over the 4 days was studied for both IGF I and ACTH.

Initiation of steroidogenesis was not as prompt under the influence of IGF I as it was with ACTH. Following an initial latent period, perhaps during which there are changes in the activities of enzymes involved in steroidogenesis, effects were apparent after 48 h of culture, with considerable increases after 72 and 96 h. In contrast with the case of ACTH, where the stimulation of 17α-hydroxylase is long-lasting and sustained [12, 16], as evidenced by the continuous increase in cortisol levels, but where corticosterone levels are diminished [12, 16, 17], IGF I was seen to provoke simultaneous increases in cortisol and corticosterone. The level of cortisol production was slightly higher and increased towards the end of the period investigated. In the experiments where the medium was collected only after 4 days' culture, the cortisol/corticosterone ratio remained elevated, which strongly suggests that IGF I is capable, not only of increasing steroidogenesis in general, but also of activating 17α-hydroxylase, a key enzyme in cortisol synthesis. This property had previously been considered peculiar to ACTH, and it is true that in our experiments the ACTH-induced increase in the cortisol/corticosterone ratio was very much more marked than that seen with IGF I. Androstenedione production was also increased by IGF I, to a degree similar to that induced by 0.25 ng/ml ACTH. Generation of androgen reflects the involvement of 17–20 lyase activity and provides further support for the involvement of 17α-hydroxylase, since the two reactions have been shown to be co-induced by the same active site of a single enzyme [18, 19]. The present findings show that IGF I on its own is capable of inducing differentiated steroidogenesis. They also substantiate the previously described potentiating capacity of IGF I on the effects of ACTH [3]. It would seem that it is

Table 2. Androstenedione production obtained after 4 days culture in serum-free McCoy's medium in the presence of a single dose of ACTH (0.25 ng/ml), IGF I (40 ng/ml), ACTH + IGF I, or insulin (300 ng/ml)

Controls	ACTH (0.25 ng/ml)	IGF I (40 ng/ml)	ACTH + IGF I	Insulin (300 ng/ml)
<1	2.2	2.4		1.7
<1	1.4	2.4		1.7
<1	2.7	2.3	7.8	

Androstenedione production (expressed as ng per 10^6 cells) was measured in 3 of the 9 experiments shown in Table 1.

Table 3. Cell count and glucocorticoid production determined in the same experiment done in quintuplicate

	Controls	ACTH (0.25 ng/ml)	IGF I (40 ng/ml)	Insulin (300 ng/ml)
No. of cells	373	406	366	410
SEM × 10^3	±26	±12	±16	±21
		($n = 5$)		
Corresp. steroid production	65	207	177	73
SEM (ng/ml)	±2	±12	±4	±3
		($n = 5$)		

Cells were seeded at 4 × 10^5 cells in 24-well plates in 1 ml McCoy's medium with 10% foetal calf serum and incubated at 37°C with 5% CO_2 for 48 h. Cell counts were done after 4 days culture in serum-free McCoy's medium in the presence of the peptide indicated.

cortisol synthesis which is amplified by the combined effects of the two peptides since, on average, the cortisol/corticosterone ratio doubled, even if only 1 out of 4 values was significantly higher than that obtained with ACTH alone.

Small doses of insulin or IGF I have been reported to have mitogenic effects on bovine adrenocortical cells [20,21]. However, under our experimental conditions of high initial seeding density and absence of extracellular matrix, HDL and transferrin in the culture medium, there was no increase in cell multiplication, and [^{3}H]thymidine incorporation into DNA was only doubled with IGF I. Furthermore, in the presence of ACTH, it was reduced. There can therefore be no link between the steroidogenic effects of IGF I and any mitogenic effect which it may have. The question arises whether the positive effects of IGF I on steroidogenesis could be attributed to an insulin-like effect. IGF I and insulin receptors [4, 22] have been identified on bovine adrenocortical cells, and the effects of both peptides result in a generalized increase in enzymatic activity [4, 23]. However, in our experiments, concentrations of insulin greatly exceeding physiological levels and equalling ten times molar concentrations of IGF I were required to obtain even slight effects on glucocorticoid production. In addition, the cortisol/corticosterone ratio remained unchanged with insulin, but increased with IGF I. IGF I is therefore capable of inducing steroidogenesis on its own, even to the extent of cortisol and androstenedione formation. It has recently been shown that this growth factor is secreted in the adrenals and also that it is regulated by ACTH and angiotensin II [24]. It is therefore possible that *in vivo* there is an interplay between the autocrine/paracrine and the endocrine actions of IGF I in maintaining the differentiated functions of the adrenocortical cell.

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