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DIFFERENTIAL MODULATION OF ACTH-STIMULATED CORTISOL AND ANDROSTENEDIONE SECRETION BY INSULIN*

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Summary—Results of previous clinical studies suggested counter regulatory actions between insulin and DHEA(S). The present studies were performed using primary monolayer cultures of bovine fasciculata-reticularis cells to test the hypothesis that insulin directly affects adrenal androgen secretion. Although having no independent effect, insulin exhibited complex time- and concentration-specific actions on ACTH-stimulated secretion of both C21 (cortisol) and C19 (androstenedione) corticosteroids. In the presence of low concentrations (0.05–0.1 nM) of ACTH, cortisol secretion during a 2 h incubation was about 2-fold greater in the presence than in the absence of insulin (0.01–100 ng/ml). In the presence of a maximal concentration (10 nM) of ACTH, on the other hand, cortisol secretion was not affected by insulin at concentrations $\leqslant 0.1$ ng/ml, but was decreased at higher insulin concentrations. ACTH-stimulated androstenedione secretion was not significantly affected by insulin during a short-term (2 h) incubation. During a prolonged (24 h) incubation, insulin produced a concentration-dependent inhibition of ACTH-stimulated cortisol secretion. At an insulin concentration of 100 ng/ml, ACTH (10 nM)-stimulated cortisol secretion declined to a level only 30% of that produced by ACTH alone. In contrast, insulin exhibited biphasic effects on the secretion of androstenedione by cells maintained in the presence of ACTH for 24 h; an effect that was most dramatic in the presence of a maximal concentration of ACTH. At an insulin concentration of 0.1 ng/ml, androstenedione secretion by cells maintained in the presence of 10 nM ACTH was increased approximately 2.5-fold. At higher concentrations of insulin, ACTH-stimulated androstenedione secretion was inhibited to an extent comparable to that in cortisol secretion. The effects of insulin on ACTH-stimulated cortisol and androstenedione secretion could not be accounted for by changes in steroid degradation or a loss in 11β-hydroxylase activity. These results indicate that insulin interacts with ACTH to modulate the secretion of both C21 and C19 corticosteroids and that physiological concentrations ($\leqslant 1$ ng/ml) of insulin may have a long-term effect to enhance selectively adrenal androgen secretion. These data are consistent with a servo mechanism between insulin and DHEA(S) *in vivo* and indicate that the correlations observed clinically result, at least in part, from a direct action of insulin to modulate the rate of adrenal androgen production.

INTRODUCTION

The adrenal cortex synthesizes and secretes a variety of C21 and C19 steroids [1–3]. In the human, adrenal androgens, particularly dehydroepiandrosterone (DHEA) and its sulfate derivative (DHEAS), are secreted in amounts equivalent to or greater than that of cortisol. It has generally been accepted that adrenal androgens are physiologically significant only as precursors for the peripheral formation of estrogens and more potent androgens. However, recent studies indicate that adrenal androgens may have more diverse and important biological actions [for review, see 4]. For example, in mice DHEA inhibited the development of both spontaneous and chemical-induced tumors [5–10]. DHEA has also been reported to inhibit both basal and stimulated mitogenesis as well as transformation/differentiation in a variety of cell lines [11–13]. In an hepatic epithelial cell line DHEA prevented the inhibition of cell growth caused by aflatoxin [14]. In genetically obese rats and mice, DHEA administration decreased insulin

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Abbreviations: DHEA, dehydroepiandrosterone; DHEAS, dehydroeipandrosterone sulfate; HBSS, Hank's Balanced Salt Solution; DNase, deoxyribonuclease; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; DMSO, dimethylsulfoxide; BHA, butylated hydroxyanisole; LDL, low density lipoprotein; ACTH, adrenocorticotropin; AG, aminoglutethimide; PDD, 4-pregnene-11β,17α-diol-3,20-dione; Δ^4-A, androstenedione; HPLC, high-performance liquid chromatography.

resistance and caused a reduction in body weight that was not fully attributable to a reduction in food intake [15–18]. In addition, DHEA appears to prevent destruction of the pancreatic β-cell and ameliorate the onset of diabetes in genetically diabetic mice [19, 20]. DHEA has also been reported to prevent, at least in part, the sequelae associated with aging and the development of autoimmune diseases in rodents [21–24].

A number of clinical observations suggest that DHEA(S) has anti-tumor, anti-obesity and anti-diabetic actions in humans. Decreased plasma and urinary levels of DHEA(S) have been correlated with an increased incidence of various malignancies [25–30]. Barrett-Connor *et al.*[31] noted an inverse relationship between serum levels of DHEAS and age-adjusted relative risk for death from cardiovascular or ischemic heart disease. Moreover, administration of DHEA to normal men increased serum high density lipoprotein levels and decreased those of low density lipoprotein [32]. Body fat content was also reduced, while insulin sensitivity remained unchanged. An inverse relationship between fasting plasma levels of DHEAS and immunoreactive insulin has been noted in hyperandrogenic women [33, 34] and in women with insulin resistance [34]. In addition, Nestler *et al.*[35] reported that hyperinsulinemia during an euglycemic clamp caused a fall in plasma DHEAS levels in normal women and one woman with hyperandrogenism and acanthosis nigricans.

These findings have renewed interest in the physiological significance of DHEA(S) as well as the identity of factors that may contribute to the regulation of adrenal androgen secretion. Results of studies demonstrating a negative correlation between serum insulin and DHEAS levels indicate that insulin may be one such factor. Furthermore, they suggest that insulin acts to decrease the metabolic clearance rate and/or production rate of DHEA(S). The studies reported here were performed using primary monolayer cultures of bovine adrenal fasciculata-reticularis cells to examine the possibility that insulin directly affects androgen secretion. The results indicate that insulin exhibits complex time- and concentration-specific interactions with ACTH in the control of corticosteroid secretion. Furthermore, the data suggest that at physiological concentrations insulin selectively promotes androgen secretion from the adrenal cortex.

EXPERIMENTAL

Cell culture

Bovine adrenal glands were collected at a local packing plant and transported to the laboratory in cold saline. Except where otherwise indicated, subsequent tissue preparation and cell isolation were performed at room temperature under aseptic conditions. Adrenals were cleaned of adhering connective tissue and transferred to Hank's Balanced Salt Solution, HBSS, containing penicillin (1000 U/ml) and streptomycin (1 mg/ml). Each gland was bisected, and medullary tissue was removed. Inner cortical (fasciculata-reticularis) tissue was then removed so that a thin layer of cells remained attached to the capsule. Adrenal capsules with adhering cells were disgarded. Fasciculata-reticularis tissue from 8–10 adrenal glands was pooled, minced, washed in HBSS and subjected to enzymatic digestion and mechanical dispersion [36, 37]. Briefly, tissue fragments were incubated at 37°C for 90 min on an orbital shaker in HBSS containing fatty acid free BSA (2 mg/ml), collagenase (type A, 4 mg/ml), DNase (0.5 mg/ml), penicillin (250 U/ml), streptomycin (250 μg/ml) and Hepes (25 mM, pH 7.4) buffer. Undigested tissue was then allowed to settle, the medium was decanted, and the tissue fragments were mechanically dispersed by pipeting. The medium and dispersed fragments were recombined, diluted 2-fold in HBSS and decanted through cheese cloth. Isolated cells were collected by centrifugation (100 *g* for 5 min). Cells were washed by centrifugation twice in HBSS and once in HBSS containing 10% horse serum (v/v). The final cell pellet was resuspended in Ham's F-12 medium containing horse serum (10%), penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (1 μg/ml), DMSO (14 mM), BHA (50 μM), α-tocopherol (1.2 μM), ascorbate (100 μM), Na_2SeO_3 (0.05 μM), glutathione (0.3 μM), insulin (10 ng/ml), transferrin (10 μg/ml), metyrapone (5 μM), $CaCl_2$ (1.8 mM), d-valine (0.1 mM), Na_2HCO_3 (4 mM), and Hepes (25 mM, pH 7.4). Antioxidants and cofactors were added for the most part at concentrations defined by Simonian *et al.*[38]. Cells were then seeded to fibronectin-coated (2 $\mu g/cm^2$) 12 well-cluster tissue culture dishes and maintained at 37°C in a humidified atmosphere of air maintained at a flow rate of 1 l/min. Medium was replaced after 16–20 h and then at 48 h intervals. Confluence was achieved within 5–7 days. At confluence, cell monolayers were incubated for an additional 24–36 h in F-12/Hepes medium supplemented with low density lipoprotein, (LDL, 10 μg protein/ml) and protease-free BSA (200 μg/ml), but in the absence of serum, insulin, and transferrin.

Ham's F12 medium was purchased from Whitaker M. A. Bioproducts (Walkersville, Md). HBSS, horse serum and antibiotic/antimycotic solutions were obtained from Grand Island Biological Company (Grand Island, N.Y.). Hepes, collagenase (Type A) and protease-free BSA were obtained from Boehringer Mannheim (Indianapolis, Ind.). Other components of the cell culture media and reagents used for the preparation of the various buffers employed in these studies were from Sigma Chemical Company (St Louis, Mo.). Tissue culture dishes were from Co-Star (Cambridge, Mass). Other plasticware was obtained from Bellco Biotechnology (Vineland, N.J.).

Design and steroid measurements

Confluent monolayers previously maintained in serum- and insulin-free medium were first incubated for two 30 min periods in the same medium, but in the absence of metyrapone. During subsequent experimental periods, cells were incubated in serum- and metyrapone-free F-12 medium containing LDL (10 μg/ml) and protease-free BSA (200 μg/ml). ACTH ($ACTH_{1-24}$, Cortrosyn, Organon Inc., West Orange, N.J.) and insulin (bovine insulin, Sigma) were added to the concentrations indicated for specific experiments. In a preliminary experiment, bovine insulin from Sigma and monocomponent porcine insulin (lot 187325) from Eli Lilly and Co. had comparable effects. Media were collected 2 h after the onset of hormone treatment, replaced with identical media, and cells incubated for an additional 24 h. Monolayers were then washed twice with cold saline and dissolved in 0.5 N NaOH. Protein contents of the cell lysates were determined by the method of Bradford[39]. Steroid contents of the incubation media were determined by radioimmunoassay using commercially available antisera (Endocrine Sciences, Tarzana, Calif.). Aliquots of incubation media containing tracer quantities of the appropriate tritiated-steroid were extracted with 1:1 (cortisol) or 3:1 (DHEA and androstenedione) hexane:ethylacetate (v/v). Radioimmunoassays were then performed as previously described [40, 41].

11β-Hydroxylase activity was assayed as the rate of conversion of exogenous 11-deoxycortisol to cortisol by cells incubated in the presence of aminoglutethimide (AG, Ciba-Geigy Corp., Summit, N.J.). Cell monolayers were treated with ACTH and/or insulin as indicated for 24 h. Cells were then incubated for 15 minutes in media containing 0.5 mM AG and, finally, for 2 h in media containing AG and 11-deoxycortisol (30 μM). The incubation was ended by transferring the monolayers to ice and rapidly removing the media. After addition of 4-pregnene-11β,17α-diol-3,20-dione (PDD, 2.5 μg), each sample was applied to a C_{18} Sep Pak cartridge (Waters, Milford, Mass) and washed with 10 ml water. Steroids were then eluted with 5 ml methanol, and the eluant evaporated under nitrogen. Steroids were redissolved in 200 μl methanol and diluted with an equal volume of water. Samples were applied to a Waters Nova Pak C_{18} (5 μ) radial pak cartridge (0.8 × 10 cm), and 11-deoxycortisol, cortisol and PDD resolved by high-performance liquid chromatography (HPLC) in a system of 55% methanol flowing at a rate of 2 ml/min. Steroids were quantitated by comparison of absorbance at 254 nm against that of authentic standards.

The metabolism of cortisol and androstenedione was examined by measuring the rate of disappearance of exogenous substrate. After a 15 min preincubation in the presence of 0.5 mM AG, cells were incubated for 24 h in the absence or presence of ACTH (0.05 or 10 nM) and/or insulin (0.01–100 ng/ml) in fresh media containing AG and either cortisol (28 μM) or androstenedione (35 μM). Media were then collected, PDD added as an internal standard, and residual substrate determined by u.v. absorbance following reversed phase HPLC. Cortisol was also measured by acid fluorescence [42] without a discernable effect on the results.

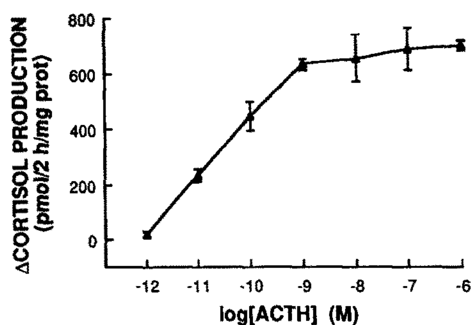

Fig. 1. Dose–response relationship between ACTH and cortisol production. Cell monolayers were incubated for 2 h in the absence or presence of increasing concentrations of ACTH (10^{-12}–10^{-6} M). Cortisol production by cells incubated in the absence of ACTH was subtracted from that by cells incubated in the presence of the hormone. Values represent the mean ± SEM of triplicate monolayers from a single experiment.

The pathway for androstenedione formation was also tentatively identified using cells incubated with AG and exogenous substrate. After a 15 min preincubation with 0.5 mM AG, cells were incubated for 24 h in fresh medium containing AG and 30 μM DHEA, 17α-hydroxypregnenolone or 17α-hydroxyprogesterone in the absence or presence of ACTH (0.05 or 10 nM) and/or insulin (0.01–100 ng/ml). Media were then removed, and cortisol and androstenedione contents determined by either u.v. absorbance after resolution by HPLC or radioimmunoassay following extraction with hexane:ethyl acetate.

Statistical analysis

Unless otherwise indicated, data represent the results of 3–6 experiments with 3 replicate monolayers per experiment. Because of variations in sensitivity and responsiveness between different cell preparations, steroid secretion rates in each experiment were normalized to a percent of that achieved in response to a maximal concentration of ACTH. Results were compared by analysis of variance and significantly ($P < 0.05$) different means identified by the method of least significant difference.

RESULTS

Basal rates of cortisol secretion by primary cultures of bovine fasciculata-reticularis cells from 6 different preparations ranged from 5.2 to 50.1 pmol × 2 h^{-1} × mg^{-1} protein with a mean ± SEM of 33.7 ± 4.3 pmol × 2 h^{-1} × mg^{-1} protein ($n = 18$). As

Table 1. Substrate preference for androstenedione formation

Substrate	Product (nmol/24 h/mg protein) Androstenedione	Cortisol
DHEA	139.60 ± 30.14	ND
17α-Hydroxypregnenolone	0.30 ± 0.01	104.23 ± 8.72
17α-Hydroxyprogesterone	0.30 ± 0.01	95.67 ± 5.65

Cells were incubated in the presence of 0.5 mM aminoglutethimide and 30 μM substrate for 24 h, and the formation of cortisol and androstenedione determined as described in Experimental. Values represent the mean ± SEM of triplicate incubations from a single experiment. The experiment was performed twice with similar results. ND indicates that the steroid was not determined.

expected, ACTH increased the rate of cortisol secretion in a concentration-dependent manner. In the experiment presented in Fig. 1, cortisol secretion by cells maintained for 2 h in the absence of ACTH was 21.5 ± 2.0 pmol $\times$ 2 h^{-1} $\times$ mg^{-1} protein. Half-maximal stimulation of cortisol secretion was obtained at a concentration of ACTH of about 0.05 nM, and maximal stimulation was achieved at a concentration of 1 nM. Although ACTH consistently increased cortisol output, sensitivity to the hormone varied substantially between cell preparations. In addition, variability in responsiveness to ACTH was particularly evident during the initial 2 h of hormone treatment, but less evident during more prolonged (24 h) incubations. For example, maximal cortisol secretion in response to ACTH (10 nM) during a 2 h incubation ranged from 233.0 to 1987.8 pmol $\times$ 2 h^{-1} $\times$ mg^{-1} protein with a mean $\pm$ SEM of 801.2 ± 196.2; that during a 24 h incubation was 110.7 ± 2.8 nmol $\times$ 24 h^{-1} $\times$ mg^{-1} protein with a range of 89.3–119.2.

ACTH also produced concentration-dependent increases in the secretion of DHEA and androstenedione. Rates of androstenedione secretion were generally less than 10% of those of cortisol in both the absence and presence of ACTH. DHEA, although secreted in amounts comparable to those of androstenedione in the absence of ACTH, was present, when detected, in amounts at least 6-fold less than that of androstenedione in the presence of ACTH. Sulfation of DHEA and, consequently, decreased recognition by the antibody employed did not account for its low rate of secretion, since comparable levels were noted using an antibody that recognized DHEA and DHEA(S) equally (data not shown). DHEA appeared to be the immediate precursor for androstenedione (Table 1). Although readily converted to cortisol, neither 17α-hydroxypregnenolone nor 17α-hydroxyprogesterone yielded significant amounts of androstenedione when provided as substrate. The preference for substrate for androstenedione formation was not affected by the presence of ACTH or insulin (data not shown).

The effects of insulin on basal and ACTH-stimulated secretion rates of cortisol and androstenedione during a 2 h incubation are presented in Fig. 2. As noted in panel A, basal rates of cortisol secretion were not affected by insulin at concentrations ranging from 0.01 to 100 ng/ml. Cortisol secretion in response to a low concentration (0.05–0.1 nM) of ACTH, on the other hand, was enhanced by insulin at a concentration as low as 0.01 ng/ml. Increasing concentrations of insulin had no additional effect

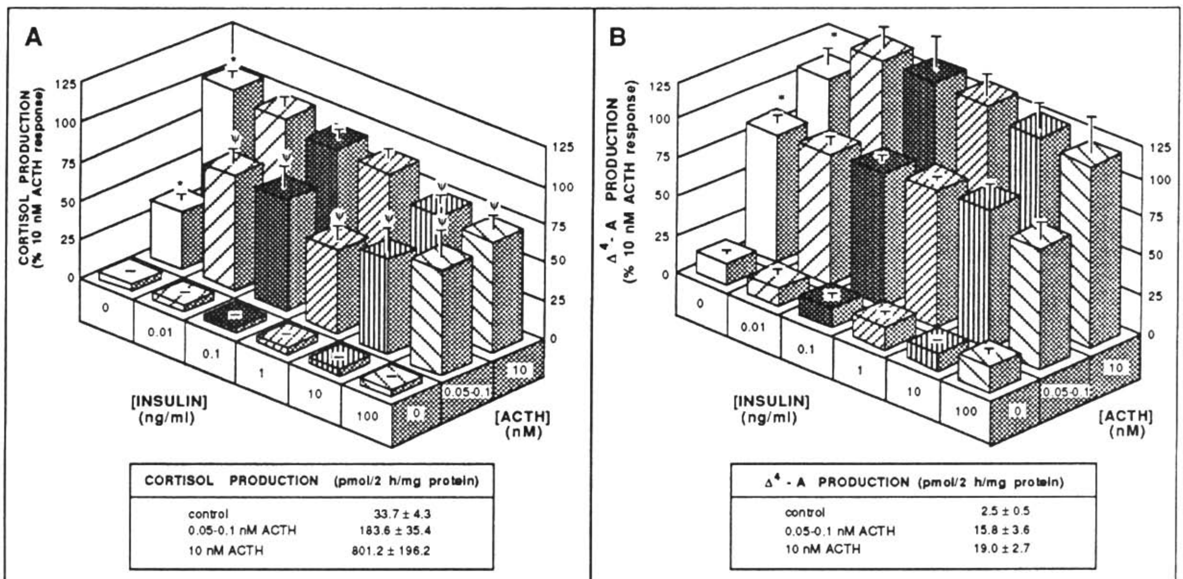

Fig. 2. Short-term effects of insulin on basal and ACTH-stimulated cortisol and androstenedione secretion. Cells were incubated for 2 h in the absence or presence of ACTH (0.05–0.1 nM or 10 nM) and/or insulin (0.01, 0.1, 1, 10 or 100 ng/ml). Cortisol (Panel A) and androstenedione, Δ^4-A, (Panel B) contents of the media were determined by RIA and normalized to a percent of the maximal (10 nM) ACTH response. Data represent the mean ± SEM of 3–9 determinations from 3 experiments. Absolute values for basal and ACTH-stimulated steroid secretion are presented in the corresponding tables. *$P < 0.05$ vs that in the absence of both ACTH and insulin. ψ$P < 0.05$ vs that in the presence of the same concentration of ACTH alone.

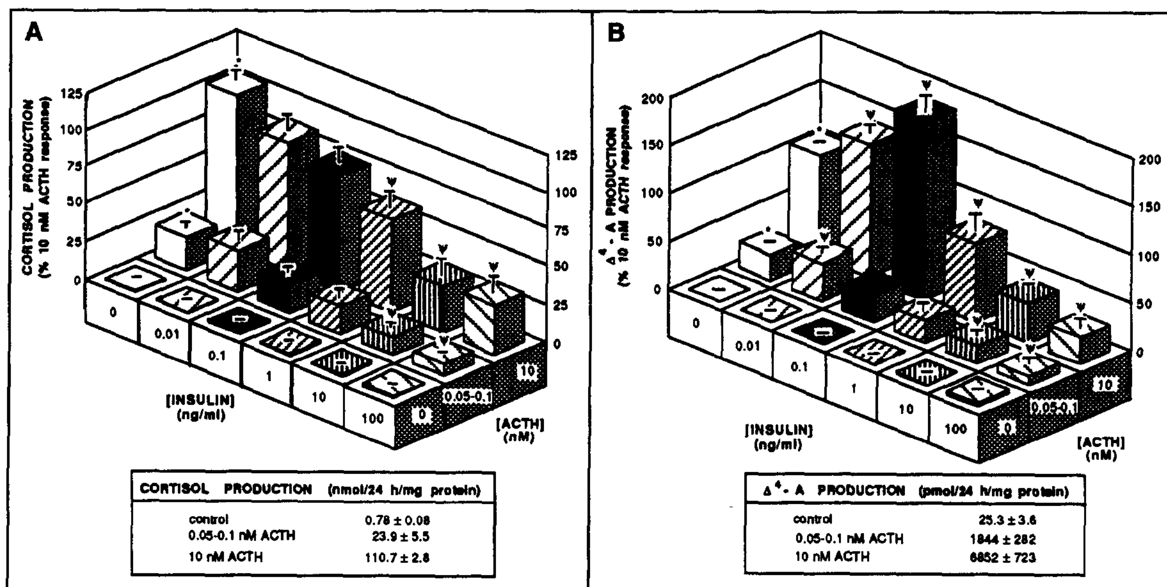

Fig. 3. Long-term effects of insulin on basal and ACTH-stimulated corticosteroid secretion. Monolayers were incubated for 24 h in the absence or presence of ACTH (0.05 or 10 nM) and/or insulin (0.01, 0.1, 1, 10 or 100 ng/ml). Cortisol (Panel A) and androstenedione, Δ^4-A, (Panel B) contents of the media were determined by RIA and normalized to a percentage of the maximal (10 nM) ACTH response. Absolute values for basal and ACTH-stimulated rates of steroid production are presented in the corresponding tables. Data represent the mean ± SEM of 9–18 determinations from 6 experiments. $^*P < 0.05$ vs that in the absence of ACTH and insulin. $^{\psi}P < 0.05$ vs that in the presence of the same concentration of ACTH alone.

on cortisol secretion. Cortisol secretion in response to a maximal concentration (10 nM) of ACTH was not affected by insulin at low concentrations (0.01–1 ng/ml), but was decreased approximately 30% by higher concentrations (10 and 100 ng/ml) of insulin. In contrast to its effects on ACTH-stimulated cortisol secretion, insulin had no significant effect on ACTH-stimulated androstenedione secretion during a 2 h incubation (panel B). Although, androstenedione secretion rates by cells maintained in the presence of 10 nM ACTH and low concentrations (0.01 and 0.1 ng/ml) of insulin tended to be greater than those by cells maintained only in the presence of ACTH, the differences were not significant with the available data. Basal rates of androstenedione secretion were also not significantly affected by insulin.

Cortisol and androstenedione secretion rates by cells maintained in the absence or presence of ACTH and/or insulin for 24 h are presented in Fig. 3. Insulin had no effect in the absence of ACTH, but had marked concentration-dependent effects on corticosteroid secretion rates in the presence of ACTH. As presented in panel A, cortisol secretion in response to low concentrations (0.05–0.1 nM) of ACTH was not affected by insulin at concentrations of 0.01–1 ng/ml, but was decreased approximately 50% at higher insulin concentrations (10 and 100 ng/ml). Cortisol secretion in response to a maximal concentration (10 nM) of ACTH was progressively inhibited by increasing concentrations of insulin. At a concentration of insulin of 10 ng/ml, a level that caused a maximal inhibition, the rate of cortisol secretion had declined to about 30% of that in the presence of ACTH alone. In contrast, insulin exhibited biphasic effects on ACTH-stimulated androstenedione secretion rates (Panel B). At an ACTH concentration of 0.05–0.1 nM, androstenedione secretion was enhanced by insulin at a concentration of 0.01 ng/ml, not affected at an insulin concentration of 0.1 ng/ml, and decreased at higher concentrations. Similarly, low concentrations (0.01 and 0.1 ng/ml) of insulin markedly increased androstenedione secretion in response to 10 nM ACTH. Maximal stimulation was achieved at an insulin concentration of 0.1 ng/ml. In the presence of higher concentrations (1–100 ng/ml) of insulin, ACTH (10 nM)-stimulated androstenedione secretion was decreased in a dose-dependent manner. At an insulin concentration of 100 ng/ml, androstenedione secretion was only 20–30% of that produced by ACTH in the absence of insulin. The effects of insulin on ACTH-stimulated DHEA secretion generally paralleled those on ACTH-stimulated cortisol secretion (data not shown).

The possibility that increased activity of degradative enzyme(s) contributed to the general decline in ACTH-stimulated corticosteroid secretion produced by insulin was examined in cells maintained in the presence of aminoglutethimide and exogenous cortisol or androstenedione. As shown in Fig. 4, both cortisol (panel A) and androstenedione (panel B) were metabolized at significant rates. The metabolism of cortisol appeared to reflect primarily

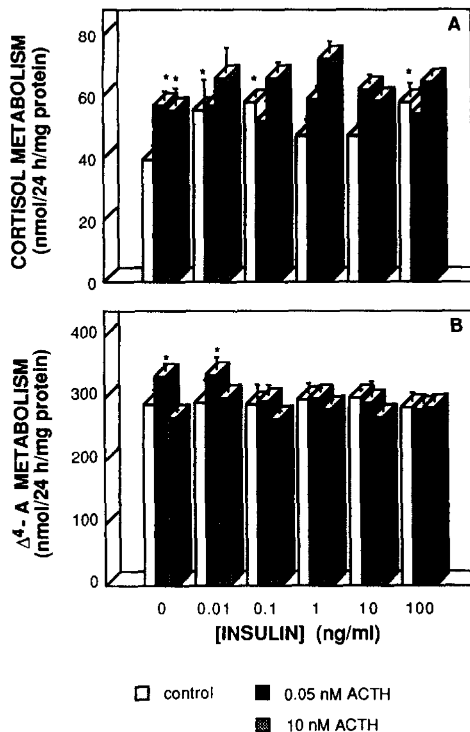

Fig. 4. Effects of insulin and ACTH on cortisol and androstenedione metabolism. Monolayers were incubated for 24 h in the presence of 0.5 mM aminoglutethimide and either exogenous cortisol (28 μM) or Δ^4-A (35 μM) without or with ACTH (0.05 nM or 10 nM) and/or insulin (0.01–100 ng/ml) as indicated. Residual substrate was then determined as described in Experimental. Values are expressed as nmols of substrate metabolized per 24 h per mg protein and represent the mean $\pm$ SEM of quadruplicate incubations from a single experiment. The experiment was performed twice with similar results. $*P < 0.05$ vs that in absence of both insulin and ACTH.

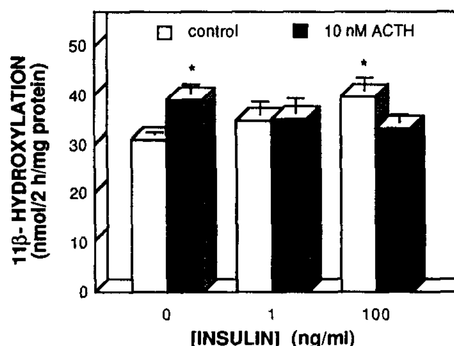

Fig. 5. Effects of insulin and ACTH on 11β-hydroxylase activity. Cells were incubated for 24 h in the absence or presence of ACTH (10 nM) without or with insulin (1 or 100 ng/ml). Media were then replaced with the same media containing 0.5 mM aminoglutethimide and exogenous 11-deoxycortisol (30 μM), and the incubation continued for 2 h. Steroids in the incubation media were then resolved by HPLC and quantitated by absorbance at 254 nm. Values represent the mean $\pm$ SEM of quadruplicate incubations from a single experiment. $*P < 0.05$ vs that in the absence of both insulin and ACTH.

5α-reductase activity, since comparable results were noted when residual cortisol was detected by acid fluorescence (data not shown). The metabolism of androstenedione, on the other hand, reflected both 5α-reduction and hydroxylation to more polar derivatives. The formation of 11β-hydroxyandrostenedione accounted for about one-third of the metabolism of androstenedione, and a number of other minor u.v.-absorbing compounds that may account for another 10–15% of its metabolism were also detected by HPLC when androstenedione was provided as substrate. Neither insulin nor a high concentration (10 nM) of ACTH, alone or in combination, affected the rate of androstenedione metabolism. Androstenedione metabolism was increased slightly by 0.05 nM ACTH, but this effect was antagonized by insulin at concentrations $\geq$0.1 ng/ml. Cortisol metabolism was increased approximately 40% by ACTH at concentrations of 0.05 nM and 10 nM. At low (0.01 and 0.1 ng/ml) and high (100 ng/ml), but not intermediate (1 and 10 ng/ml), concentrations, insulin increased the rate of cortisol metabolism to a level comparable to that achieved in response to ACTH. In the presence of both ACTH and insulin, however, the rate of cortisol metabolism was no greater than that in the presence of either hormone alone.

Experiments were also performed to address the possibility that a loss of 11β-hydroxylase contributed to the insulin-induced decline in ACTH-stimulated cortisol secretion [43, 44]. As noted in Fig. 5, 11β-hydroxylase activity in cells maintained in the presence of either ACTH (10 nM) or insulin (100 ng/ml) was approximately 30% greater than that in cells maintained in the absence of either hormone. Although the independent effects of ACTH and insulin were abolished when the hormones were present simultaneously, 11β-hydroxylase activity remained at a level comparable to that of control cells.

DISCUSSION

There have been numerous attempts to identify a hormone in addition to ACTH that would stimulate androgen secretion by the adrenal cortex. With the noted exception of a peptide recently identified by Parker *et al.*[45], no other pituitary or placental hormone thus far examined selectively increased androgen secretion [46–48]. Androgen secretion by cultured cells of ovarian or testicular origin has been shown to be enhanced by insulin [49–52], and considerable evidence, albeit indirect, has been presented suggesting that insulin may also participate in the control of adrenal androgen secretion [33–35]. The results of the present studies indicate directly that insulin exerts effects on cultured bovine fasciculata-reticularis cells to differentially enhance the secretion of C21 and C19 steroids.

The effect of insulin to enhance cortisol secretion in response to low concentrations of ACTH is consistent with its effect on corticosteroid secretion noted

in previous studies [53, 54]. However, the results of the present studies and those of other investigators are not directly comparable. Penhoat *et al.*[53] noted an increase in corticosterone secretion in response to ACTH (1 nM) after a 72 h pretreatment with high concentrations (50 ng/ml or 10 μg/ml) of insulin. Similarly, although effects of insulin were apparent within 4 h, cholera toxin-stimulated LDL metabolism and 11-deoxycortisol secretion were augmented by insulin in a time- (0–20 h) and concentration-specific (1–10,000 ng/ml) manner [54]. The enhancement of ACTH-stimulated cortisol secretion reported here, in contrast, appears to reflect an acute action of insulin and be of relatively short duration. In addition, the stimulatory effect of insulin on cortisol secretion was apparent only in the presence of submaximal concentrations of ACTH. In the presence of a maximal concentration (10 nM) of ACTH or with more prolonged (24 h) treatment, the predominant effect of insulin on ACTH-stimulated cortisol secretion was a concentration-dependent inhibition.

The reasons for these discrepancies in the apparent actions of insulin on C21 steroid secretion are unclear. The differences in the effects of the peptide on the rates of secretion of 11-deoxycortisol [54] and cortisol (Figs 2 and 3) can not be accounted for by a decrease in 11β-hydroxylase activity. Nor can differences in cell type readily explain those in the actions of insulin, since bovine adrenocortical cells were employed in this as well as the previous studies. Differences in the conditions under which the cells were maintained, however, may determine their relative responsiveness to secretagogues, and our data would suggest that sensitivity and responsiveness to ACTH determines, at least in part, the direction and magnitude of the effect produced by a given concentration of insulin. It is unclear whether parameters influencing the steroidogenic capability of the cells (i.e. whether the cells secrete cortisol, 11-deoxycortisol or corticosterone) would also alter their response to hormone action or interaction. In addition, the effects noted on corticosteroid secretion in response to the high concentrations ($>$ 100 ng/ml) of insulin employed in previous studies may reflect, in part, down regulation of the insulin receptor and interaction with the type 1 insulin-like growth factor receptor [51, 53]. The divergent effects of insulin on ACTH-stimulated cortisol secretion noted in the present study, in contrast, were apparent at physiological concentrations and most likely reflect actions exerted through the insulin receptor. In any event, the results of the present studies indicate that the physiological effects of insulin on the secretion of C21 corticosteroids are more complex than previously reported.

The results of the present study also indicate that insulin at physiological concentrations and in the presence of ACTH exerts complex effects to modulate androgen secretion by the adrenocortical cell. Moreover, insulin exhibited time- and concentration-dependent interactions with ACTH to promote the secretion of androstenedione that were distinct from those resulting in an increase in cortisol secretion. In contrast to its effect to increase ACTH-stimulated cortisol secretion, insulin had no significant acute (2 h) effect on ACTH-stimulated androstenedione secretion. During more prolonged (24 h) treatment, insulin exhibited concentration-specific interactions with ACTH to enhance or inhibit the secretion of androstenedione. The concentrations (0.01–0.1 ng/ml) of insulin that enhanced ACTH-stimulated androstenedione secretion also produced a 25–30% decrease in ACTH-stimulated cortisol secretion. The effect of insulin to increase ACTH-stimulated androstenedione secretion, in further contrast to its effect to increase ACTH-stimulated cortisol secretion, was most dramatic in the presence of a maximal concentration of ACTH. These observations suggest that insulin and ACTH may interact through a common site to promote glucocorticoid secretion, but act through different mechanisms to promote androgen secretion. At higher concentrations (1–100 ng/ml) of insulin, ACTH-stimulated androstenedione secretion progressively declined in a manner and to an extent comparable to ACTH-stimulated cortisol secretion.

There is presently little direct information concerning the mechanism(s) by which physiological concentrations of insulin modulate ACTH-stimulated secretion of C21 and C19 corticosteroids. The effects of insulin on cortisol and androstenedione secretion noted in the present studies, however, can not be adequately accounted for by changes in steroid degradation. Thus, insulin appears to be affecting parameters involved in steroid biosynthesis. The effect of insulin to enhance ACTH-stimulated cortisol secretion during short-term incubations most probably reflects an effect on cholesterol mobilization and a concomitant increase in cholesterol side chain cleavage activity. This supposition is consistent with the effects of insulin to enhance agonist-stimulated LDL uptake and degradation and to promote pregnenolone production [53, 54].

It is somewhat surprising that the acute increase in cortisol secretion was not accompanied by a comparable increase in androstenedione secretion. This may reflect the wide variation in the steroidogenic response noted at 2 h as well as the fact that the rate of androstenedione secretion was only a fraction of that of cortisol secretion. Regardless, there was also a clear difference in the ACTH dose–response relationship between cortisol and androstenedione secretion. The differential effects of insulin and ACTH on cortisol and androstenedione secretion at 2 h may well reflect a differential regulation of C21 and C19 steroid secretion. In fact, an effect of insulin to selectively enhance ACTH-stimulated androstenedione secretion was clearly apparent at 24 h.

The means by which insulin selectively enhances androstenedione secretion is presently unknown. It

has been suggested that low 3β-hydroxysteroid dehydrogenase activity relative to 17α-hydroxylase activity facilitates androgen formation [55, 56]. However, insulin has been reported to promote 3β-hydroxysteroid dehydrogenase activity [53], and in the present studies insulin had no apparent effect on the rate of conversion of DHEA to androstenedione. Thus, it seems unlikely that changes in 3β-hydroxysteroid dehydrogenase activity contribute significantly to those in androstenedione secretion. The present data suggest that the primary means by which insulin specifically enhances androstenedione secretion is to preferentially promote 17,20-lyase activity. Such an action would direct proportionately more substrate away from glucocorticoid and toward androgen formation. An increase in 17,20-lyase activity coupled with an effect of ACTH to promote substrate flux into the steroidogenic pathway could fully account for the action of insulin to selectively enhance ACTH-stimulated androstenedione secretion. The time required to demonstrate an increase in androstenedione secretion is consistent with an effect of insulin on enzyme induction. An increase in cytochrome P-450_{17} in and by itself, however, would probably not contribute to a specific increase in androgen formation, since a single gene product appears to exhibit both 17α-hydroxylase and 17,20-lyase activities [57]. Rather, an effect of insulin to increase the content of a cofactor, coenzyme or some other component that would preferentially enhance 17,20-lyase activity would be required. Additional studies are needed to specifically address these issues.

The mechanisms responsible for the general inhibition of ACTH-stimulated corticosteroid secretion noted here in response to high concentrations of or during prolonged (24 h) treatment with insulin are probably distinct from those responsible for the positive effects of the peptide. The decline in both C21 and C19 steroid secretion is consistent with desensitization to ACTH. Although not specifically addressed in the present studies, the observation [53] that prolonged (72 h) treatment with insulin at a concentration (50 ng/ml) comparable to that used here enhanced the acute cAMP response to ACTH in bovine adrenocortical cells suggests that insulin did not accelerate down regulation of the ACTH receptor or impede activation of adenylate cyclase. Actions of insulin to increase cAMP phosphodiesterase activity [58–60] and to directly oppose those of cAMP [61–63] noted in other cell types may have contributed to the attenuation of ACTH-stimulated corticosteroid secretion noted in the present studies.

The significance of an inhibitory effect of insulin on ACTH-stimulated steroidogenesis is unclear. This effect is not easily explained in light of observations suggesting that insulin has a permissive action to maintain differentiation of steroidogenic cells [49–54]. As noted previously, however, these observations may largely reflect pharmacological actions of insulin. The present data are strengthened by the fact that the effects were demonstrated at physiological concentrations and that the divergent time- and concentration-dependent actions of insulin were, for the most part, steroid specific. These data are also supported by those recently presented by Farah *et al.*[64] indicating an inverse relationship between plasma immunoreactive insulin levels and DHEA production *in vivo*. It is noteworthy that insulin did not completely abolish ACTH-stimulated corticosteroid secretion, and ACTH continued to produce clear concentration-dependent increases in corticosteroid secretion rates in the presence of insulin. In cells maintained in the presence of 100 ng/ml insulin for 24 h the steroidogenic response to 10 nM ACTH was decreased almost 70% compared to that of control cells, but secretion rates of cortisol and androstenedione were still some 35-fold greater than those of cells maintained in the presence of insulin alone. Such a response may allow for an appropriate secretion of corticosteroids under conditions of hyperinsulinemia. Alternatively, additional factors may serve to maintain the responsiveness of the adrenal cortex to ACTH *in vivo*.

In summary, the results of the present studies indicate that insulin exerts complex effects to modulate corticosteroid secretion and suggest that at normal physiological concentrations insulin may chronically modulate the actions of ACTH and perhaps other factors to preferentially enhance androgen secretion by the adrenal cortex. Moreover, these data are consistent with the existence of a regulatory mechanism between plasma levels of immunoreactive insulin and DHEA(S) [65] and provide direct evidence that the effects of insulin on DHEA(S) levels in polycystic ovarian disease and related dysfunctions reflect, at least in part, effects on the rate of adrenal DHEA(S) production.

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