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EFFECT OF CORTISOL ON TESTOSTERONE PRODUCTION BY IMMATURE PIG LEYDIG CELLS

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Summary—The direct effects of hydrocortisone (HS) and adrenocorticotropin (ACTH) on testicular testosterone production were studied in purified immature pig Leydig cells *in vitro*. Leydig cells were obtained from 3- to 4-week-old piglet testes by enzymatical dispersion followed by discontinuous Percoll gradient centrifugation. Leydig cells were treated with HS and ACTH in the absence or presence of luteinizing hormone (LH) after 12 h of incubation. Media were collected 48 h later for testosterone and cyclic adenosine 3′,5′-monophosphate (cAMP) measurement. Treatment of Leydig cells with increasing concentrations (0.001–10.0 μg/ml) of HS for 48 h resulted in a dose-dependent increase in basal and LH-stimulated testosterone production. Increasing duration (6–72 h) of treatment with HS (100 ng/ml) led to a time-dependent increase in basal and LH-stimulated testosterone production, achieving statistical significance by 48 and 24 h, respectively. HS increased LH-stimulated cAMP production. HS also increased testosterone production induced by $(Bu)_2$ cAMP. Forskolin stimulated testosterone production to an extent comparable to that attained with LH, and HS augmented forskolin-stimulated testosterone production. HS enhanced the conversion of exogenous 17α-hydroxyprogesterone to testosterone, but did not affect the conversion of pregnenolone and progesterone to testosterone, suggesting a specific stimulation of 17,20-desmolase. Porcine ACTH had no influence on basal and LH-stimulated testosterone production. These results suggest that HS directly stimulates immature pig Leydig cell steroidogenesis, at least in part via an enhancement of the generation of cAMP, leading to an increase in the activity of 17,20-desmolase.

INTRODUCTION

It is well-accepted that various stressors such as social dominance [1], confinement [2] and high ambient temperature [3–5] can influence testicular function in boars. This effect is thought to be mediated, in part, through the activation of the hypothalamic–pituitary–adrenal axis which in turn alters the brain–pituitary–gonadal axis. Changes in serum cortisol, the predominant glucocorticoid in pigs, are considered a valid indication for stress in this species [6–8]. A high level of circulating cortisol with a corresponding increase in testosterone concentration has been found in boars following acute treatment with adrenocorticotropin (ACTH) [9-12]. Chronic treatment with ACTH, however, resulted in decreased testosterone production [9]. Although luteinizing hormone (LH) is the primary hormone regulating testicular androgen biosynthesis [13, 14], the ACTH-induced increase in testosterone production by the pig testes was independent of changes in peripheral concentrations of LH [11]. The ACTH effect may be mediated through the adrenal cortex. But, a possible direct testicular effect of ACTH cannot be ruled out either. The exact mechanism by which ACTH exerts its effect is presently still not clear.

A few studies have investigated the direct action of glucocorticoids on the testicular function *in vitro*. Hipkin and Raeside [15] found a direct stimulatory effect of cortisol on testosterone production by pig Leydig cells upon acute exposure, whereas Bernier *et al.* [16] found a direct inhibitory effect of dexamethasone, a synthetic glucocorticoid, after 18 h of exposure. The inhibition of Leydig cell function by dexamethasone was also observed in the rat [17, 18]. The latter study [18] suggests that glucocorticoids may decrease both the cyclic adenosine 3′,5′-monophosphate (cAMP) response to LH stimulation and 17α-hydroxylase activity. In addition, studies demonstrating specific glucocorticoid receptors in testicular tissue have been reported [19–21]. The possibility that ACTH may have a direct effect on testicular testosterone production has been shown both *in vivo* [22]

and *in vitro* [23] in rabbits. Consequently, the objective of the present study was to further investigate the direct effect of ACTH and cortisol on testosterone production by *in vitro* pig Leydig cells upon chronic exposure. The mechanisms of action of cortisol on testosterone biosynthesis were also tested.

EXPERIMENTAL

Materials

Porcine ACTH, forskolin, dibutyryl cyclic adenosine 3′,5′-monophosphate [$(Bu)_2$cAMP], progesterone, pregnenolone, 17α-hydroxyprogesterone, insulin, human transferrin, epidermal growth factor, collagenase Type I (300 U/mg) and all other chemical were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Porcine LH (LER-778-4) was provided by Dr Leo Reichert Jr (Department of Biochemistry, The Albany Medical College of Union University, Albany, N.Y., U.S.A.). Hydrocortisone sodium succinate (HS; Solu-Cortef) was purchased from The Upjohn Co. (Kalamazoo, Mich., U.S.A). Percoll was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Cell culture media and other supplies were purchased from the Grand Island Biological Co. (Brand Island, N.Y.). Dextran-coated charcoal-absorbed serum was prepared by incubation of the serum with 1% charcoal and 0.1% dextran T-70 at 4°C for 18 h under constant stirring, followed by centrifugation and Millipore filtration (Millipore Corp., Bedford, Mass. U.S.A.).

Preparation of Leydig cells

Testes were obtained from 3- to 4-week-old piglets at the Taiwan Livestock Research Institute herd at the time of routine castration and were carried to the laboratory in chilled 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) buffer (137 mM NaCl, 5 mM KCl, 0.7 mM $NaHPO_4$, 25 mM HEPES, 10 mM glucose and 360 μM $CaCl_2$) containing 0.1% bovine serum albumin (BSA). The testes were decapsulated, sliced and washed 4 times with HEPES–0.1% BSA buffer. The testicular slices were dispersed by constant stirring for 90 min at 34°C in HEPES–0.1% BSA buffer containing 0.02% collagenase–DNAase and 0.003% trypsin inhibitor. The crude cell suspension was filtered twice through two layers of cheesecloth. The cells were then washed with phosphate buffer (PBS; 0.1 M PO_4, 0.15 M NaCl and 0.01% thimerosal, pH 7.4) containing 0.07% BSA, collected by centrifugation for 5 min at 200 *g* and resuspended in PBS–0.07% BSA. The cell suspension (5–10 × 10^7 cells/ml PBS–0.07% BSA/gradient) was layered on the top of a discontinuous Percoll gradient prepared as described by Schumacher *et al.* [24] with slight modifications. Linear density gradients (1.02–1.10 g/ml, 9 ml) were prepared by diluting the Percoll solution (9 parts Percoll and 1 part 10-fold conc. PBS–0.7% BSA) with PBS–0.07% BSA. After centrifugation at 800 *g* at 20°C for 20 min, Leydig cells were obtained in a band corresponding to densities between 1.05–1.07 g/ml, as determined by histochemical 3β-hydroxysteroid dehydrogenase staining [25], washed twice with PBS–0.07% BSA and sedimented by centrifugation at 200 *g* for 5 min at 4°C. The pooled cells were resuspended in McCoy's 5a medium (0.5 × 10^6 cells/ml) supplemented with L-glutamine (2 mM), pencillin (50 U/ml), streptomycin sulfate (50 μg/ml), fungizone (amphotericin B-250, 0.625 μg/ml), 25 mM HEPES, insulin (5 μg/ml), human transferrin (5 μg/ml) and epidermal growth factor (10 ng/ml) in the presence of 0.1% fetal calf serum. The serum was pretreated with dextran–charcoal. 2 ml of this suspension were transferred to a tissue culture dish (35 × 10 mm). Virtually all cells remained intact and viable according to trypan blue dye test. The purity of Leydig cells was >85%.

Incubation of Leydig cells

Leydig cells were maintained at 34°C in a 5% CO_2–air atmosphere and treated with ACTH or HS in the presence or absence of porcine LH after 12 h of incubation. Media were collected 48 h later, or as indicated in the Results section, and stored frozen until assayed by radioimmunoassay (RIA) for testosterone and cAMP, unless otherwise indicated. All compounds added to the culture were diluted with culture medium. The addition volume was 10 μl. The test steroids were dissolved in ethanol and then diluted with culture medium before addition to the Leydig cells. Data in this study were obtained from different preparations of cells, each being prepared from six testes. Within each preparation, there were three replicates per treatment. All data reported represented a triplicate determination of a typical experiment performed at least twice unless otherwise indicated.

Radioimmunoassays (RIAs)

Testosterone concentration in the unextracted medium was measured by RIA according to Li *et al.* [26]. The antiserum to testosterone-11–BSA was obtained from Dr G. D. Niswender (Department of Physiology, Colorado State University, Fort Collins, Colo, U.S.A.) and used at a dilution of 1:10,000 in 0.01 M Tris buffer (pH 7.4). Validation of this antiserum has been reported previously [27]. The intra-assay variation, determined by duplicates of three dose levels of the control medium from the Leydig cell pool, was <10%.

The medium concentration of cAMP was determined using Amersham's cAMP assay kit (Amersham International Plc, Amersham, England).

Determination of cell number and cellular protein content

At the completion of some experiments, the total cell number and cellular protein content were determined for the HS-treated and control groups. After the removal of the incubation media, 1.0 ml of 0.25% trypsin solution was added to each culture dish. The cells were scraped off the dish with a rubber policeman after being incubated at 34°C for 5–10 min and centrifuged at 200 *g* for 5 min. The cells of the pellet were washed twice with HEPES–0.1% BSA buffer and resuspended for counting in a hematology analyzer (Cell-Dyn 300, Sequoia-Turner Corp., Mountain View, Calif., U.S.A.). The cellular protein content was determined using the Bio-Rad protein assay kit (Bio-Rad Labs, Richmond, Calif., U.S.A) [28], using bovine plasma albumin as standard.

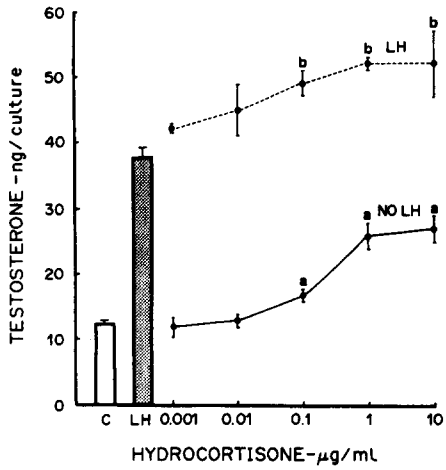

Fig. 1. Concentration-dependence of the effect of HS on testosterone production by cultured pig Leydig cells. Leydig cells were cultured with or without LH (10 ng/ml) in the absence or presence of increasing concentrations (0.001–10 μg/ml) of HS. Control cultures (C) received media alone. Testosterone levels were measured after 48-h incubations. Results are expressed as mean ± SEM ($n = 3$). Comparable results were obtained in two other experiments. ([a]$P < 0.01$ vs control values; [b]$P < 0.05$ vs LH-treated values.)

Data analysis

Two means were compared using Student's t-test. Where there were more than two means, significant differences between means were determined by analysis of variance [29]. The means were then analyzed by Duncan's multiple range test.

RESULTS

Effect of treatment with HS on testosterone production

The dose-dependence of the effect of HS on basal and LH-stimulated testosterone production is shown in Fig. 1. HS at a dose of 0.1 μg/ml or higher significantly increased basal ($P < 0.01$) and LH-stimulated ($P < 0.05$) testosterone production.

Results in Table 1 show that incubation of pig Leydig cells with a constant dose of HS (100 ng/ml) for 48-h stimulated testosterone production at least 2-fold at every dose of LH tested.

In the time-course experiment, Leydig cells were cultured with or without a constant dose of HS (100 ng/ml) in the absence or presence of LH (10 ng/ml) for 6, 24, 48 or 72 h (Fig. 2). Treatment with HS caused significant ($P < 0.05$) increases in basal and LH-stimulated testosterone production at times greater than 24 and 6 h, respectively.

Effect of treatment with HS on LH-induced extracellular cAMP production

As shown in Table 2, the medium concentration of cAMP was low in both control

Table 1. Effect of HS on LH-stimulated testosterone production by cultured pig Leydig cells[a]

	Testosterone (ng/culture)		
LH (ng/ml)	−HS	+HS	P-value
0	3.97 ± 0.27	9.62 ± 0.47	0.001
0.1	5.35 ± 0.13	14.18 ± 0.92	0.001
1.0	10.61 ± 0.04	20.83 ± 1.14	0.001
10.0	13.29 ± 0.12	26.92 ± 2.80	0.008

[a]Leydig cells were cultured with 100 ng/ml HS (+HS) or without HS (−HS) in the presence of increasing concentrations (0.1–10.0 ng/ml) of LH. Testosterone levels were measured after 48-h incubations. Results are the mean ± SEM of triplicate cultures of a typical experiment. P-values are comparisons of LH-stimulated cells with and without HS.

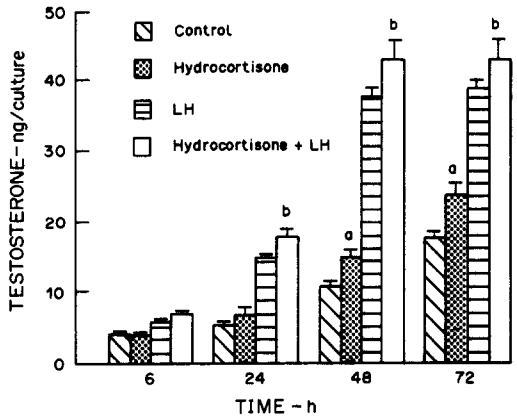

Fig. 2. Time-course of HS stimulation of testosterone production by pig Leydig cells. Leydig cells were cultured in the absence or presence of LH (10 ng/ml) with or without HS (100 ng/ml) at the designated time points. Media were collected and the testosterone released into the medium was measured. Results are expressed as mean ± SEM ($n = 3$). Similar results were observed in an additional experiment. [a]Significantly different from the corresponding time point of the control group, $P < 0.05$; [b]significantly different from the corresponding time point of the LH group, $P < 0.05$.

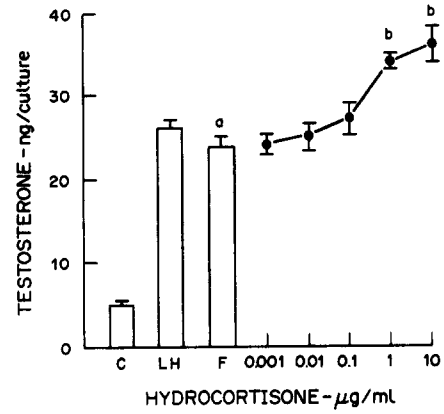

Fig. 3. Effect of HS on forskolin-stimulated testosterone production. Leydig cells were cultured with forskolin (F; 10^{-5} M) in the absence or presence of increasing concentrations (0.001-10 μg/ml) of HS. Some cells were treated with 10 ng/ml LH or without LH (controls; C). Testosterone levels were measured after 48-h incubations. Results are expressed as mean ± SEM ($n = 3$). ([a]$P < 0.01$ vs control values; [b]$P < 0.05$ vs forskolin-treated values.)

and HS-treated cells in the absence of 0.1 mM 1-methyl-3-isobutyl xanthine (MIX), a phosphodiesterase inhibitor, while LH administration significantly ($P < 0.001$) increased cAMP production as compared to controls. HS enhanced LH-stimulated cAMP production by 30% ($P < 0.001$). In the presence of the phosphodiesterase inhibitor, cAMP production was enhanced in all treatment groups. Analysis of variance revealed significant ($P < 0.001$) interactions between LH and HS and LH and MIX for extracellular cAMP production.

Effect of treatment with HS on forskolin- and $(Bu)_2$cAMP-stimulated testosterone production

The influence of HS upon the ability of forskolin, an activator of adenylate cyclase, and $(Bu)_2$cAMP to stimulate testosterone production was also investigated (Fig. 3). Administration of forskolin significantly ($P < 0.01$) stimulated testosterone production (24.23 ± 1.09 ng/culture) 5-fold relative to controls (5.08 ± 0.54 ng/culture) and comparable to that attained with LH (26.04 ± 1.30 ng/culture). HS at a concentration higher than 100 ng/ml enhanced the stimulatory effect for forskolin ($P < 0.05$).

Results in Fig. 4 show that treatment with $(Bu)_2$cAMP significantly ($P < 0.01$) increased testosterone production as compared to controls, whereas concomitant treatment with increasing concentrations of HS stimulated testosterone production in a dose-dependent manner with

Table 2. Effect of HS on basal and LH-stimulated extracellular cAMP production by cultured pig Leydig cells[a]

Treatment	Extracellular cAMP (pmol/culture)
CON	0.68 ± 0.02
CON + MIX	4.95 ± 1.55
HS	1.37 ± 0.68
HS + MIX	6.94 ± 0.80
LH	86.53 ± 3.93
LH + MIX	213.71 ± 7.99
LH + HS	112.32 ± 3.55
LH + HS + MIX	257.19 ± 0.80

[a]Leydig cells were cultured with or without HS (100 ng/ml) in the absence (CON) or presence of LH (10 ng/ml); some of the cultures contained 0.1 mM MIX. Media were collected after 48-h incubations and concentrations of cAMP were determined by RIA. Results are the mean ± SEM of triplicate cultures.

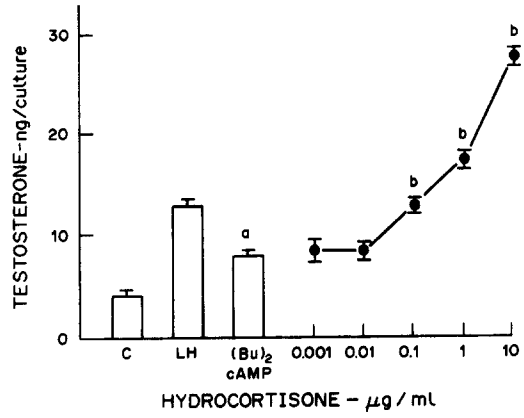

Fig. 4. Effect of HS on $(Bu)_2$cAMP-stimulated testosterone production. Leydig cells were cultured with $(Bu)_2$cAMP (50 μg/ml) in the absence or presence of increasing concentrations (0.001–10 μg/ml) of HS. Some cells were also treated with 10 ng/ml LH or without LH (controls; C). Testosterone levels were measured after 48-h incubations. Results are expressed as mean ± SEM ($n = 3$). [[a]$P < 0.01$ vs control values; [b]$P < 0.001$ vs $(Bu)_2$cAMP-treated values.]

Table 3. Effect of HS on the conversion of pregnenolone, progesterone and 17α-hydroxyprogesterone to testosterone by cultured pig Leydig cells[a]

Treatment	Testosterone (ng/culture)		
	−HS	+HS	*P*-value
Control	5.07 ± 0.39	7.99 ± 0.42	<0.01
Pregnenolone	223.05 ± 3.02	232.20 ± 5.02	NS
Progesterone	190.35 ± 7.54	194.40 ± 4.06	NS
17α-hydroxy-progesterone	382.09 ± 6.23	405.62 ± 1.96	<0.05

[a]Leydig cells were cultured either with pregnenolone (10^{-5} M), progesterone (10^{-5} M) or 17α-hydroxyprogesterone (10^{-5} M) with or without HS (100 ng/ml). Testosterone levels were measured after 48-h incubations. Results are the mean ± SEM of triplicate cultures. *P*-values were comparisons of the same treatment with and without HS (by Student's *t*-test).

10 μg/ml of HS increasing testosterone production by 263%.

Effect of treatment with HS on the conversion of exogenous pregnenolone, progesterone and 17α-hydroxyprogesterone to testosterone

To examine the site(s) in the steroidogenic pathway affected by HS, the effects on the conversion of exogenous pregnenolone, progesterone or 17α-hydroxyprogesterone to testosterone in the absence or presence of HS (100 ng/ml) were further characterized. As shown in Table 3, addition of either pregnenolone, progesterone or 17α-hydroxyprogesterone produced significant ($P < 0.001$) increases in testosterone production. In the presence of HS, a marked ($P < 0.05$) increase in testosterone production by Leydig cells was observed only when 17α-hydroxyprogesterone was added. When cells were treated either with pregnenolone or progesterone, HS did not affect the testosterone production.

Effect of treatment with HS on Leydig cell number and protein content

Whether stimulation of testosterone production by HS could be accounted for by changes in total cell number or cellular protein content was further evaluated (Table 4). Under our experimental conditions, treatment of control or LH-stimulated Leydig cells with HS (100 ng/ml) did not result in significant alterations in either cell number or protein content.

Table 4. Effect of HS treatment on Leydig cell number and cellular protein content[a]

Treatment	Cell number (1×10^6/culture)	Protein content (μg/culture)
None	1.07 ± 0.16	49.18 ± 0.84
HS	1.03 ± 0.12	46.52 ± 1.13
LH	1.25 ± 0.10	48.95 ± 1.02
LH + HS	1.13 ± 0.14	47.44 ± 1.89

[a]Leydig cells were cultured with or without LH (10 ng/ml) in the absence or presence of HS (100 ng/ml). Determinations were carried out after 48-h incubations as described under Experimental. Results are the mean ± SEM of triplicate cultures.

Table 5. Effect of porcine ACTH on testosterone production by cultured pig Leydig cells[a]

Porcine ACTH (ng/ml)	Testosterone (ng/culture)	
	−LH	+LH
0	11.72 ± 0.78	21.50 ± 1.34
10	10.76 ± 0.90	20.84 ± 1.22
50	10.80 ± 0.90	20.47 ± 1.44
100	11.77 ± 0.04	21.73 ± 1.84
500	10.41 ± 0.28	20.32 ± 1.42
1000	10.22 ± 0.62	22.68 ± 2.74

[a]Leydig cells were cultured with 10 ng/ml LH (+LH) or without LH (−LH) in the absence or presence of increasing concentrations (10–1000 ng/ml) of porcine ACTH. Testosterone levels were measured after 48-h incubations. Results are the mean ± SEM of triplicate cultures. Similar results were observed in an additonal experiment.

Effect of treatment with porcine ACTH on testosterone production

Table 5 shows the effect of porcine ACTH on basal and LH-stimulated testosterone production. Testosterone production in control cultures averaged 11.72 ± 0.78 ng/culture and was increased ($P < 0.05$) by LH stimulation. Treatment with increasing concentrations of porcine. ACTH had no influence on basal or LH-stimulated testosterone production.

DISCUSSION

The direct effects of HS on basal and LH-stimulated production of testosterone were investigated with immature pig Leydig cells. Our findings indicate that HS can enhance both basal and LH-stimulated Leydig cell steroidogenesis. The HS effect is dose- and time-dependent and is associated with increases in cAMP formation, but not associated with alterations in the total Leydig cell number or cellular protein content. Furthermore, this direct effect may be accounted for, at least in part, by selectively stimulating the reaction catalyzed by 17,20-desmolase.

HS, at the concentrations used, stimulated testosterone production when added to cultures of purified Leydig cells obtained from immature pigs for longer than 24 h of incubation. This finding is in agreement with a previous report of Hipkin and Raeside [15] in which cortisol stimulates testosterone production from mature pig Leydig cells with an *in vitro* perifusion system. However, our results contradict the earlier findings that cortisol can directly inhibit androgen biosynthesis in primary cultures of nonpurified testicular cells obtained from adult hypophysectomized rats [17, 18]. This discrepancy might be explained by the differences in experimental animal and(or) conditions used (purified Leydig

cells vs crude testicular preparations). The interaction between Sertoli and Leydig cells in the regulation of testicular steroidogenesis has been reported. Parvinen *et al.* [30] and Syed *et al.* [31] found that a factor produced by seminiferous tubules can inhibit androgen secretion in interstitial cell preparations but stimulate androgen output by purified Leydig cells. Benahmed *et al.* [32] also demonstrated that FSH stimulates the activity of Leydig cells cocultured with Sertoli cells, whereas the same hormone does not affect the activity of Leydig cells cultured alone. Should the stimulatory effect of cortisol observed in this study be mediated by a cell type other than Leydig cells, experiments utilizing cocultures of Leydig cells and Sertoli cells will be needed to clarify this point.

In our studies the minimal effect stimulatory dose of HS was at 100 ng/ml, which is comparable with that found in pigs following ACTH administration or application of an acute stressor [33], but lies above the circulating levels under nonstressed conditions. Since >90% of circulating glucocorticoids are bound by transcortin [34, 35], the present observations may represent an overestimate of the *in vivo* sustained stressed conditions and the effect seen is probably more pharmacological than physiological. It is thus possible that cortisol may not have a role in the increase in testosterone secretion which occurs following ACTH administration, as reported by Juniewicz and Johnson [33]. However, under the conditions of our experiment, the demonstration of a direct stimulatory effect of HS on testosterone production suggests that other mechanisms to diminish testicular function during stress may exist. The possibility that glucocorticoids may act directly on the target tissues of testosterone remains to be investigated.

Our studies on the mechanism of action of HS have shown that HS treatment enhances LH-stimulated cAMP production in the presence or absence of the phosphodiesterase inhibitor. Our results also show that HS can augment forskolin-stimulated testosterone production. These findings suggest that HS may stimulate adenylate cyclase activity, resulting in the generation of cAMP. In addition, to stimulate cAMP formation, our observation that HS treatment also enhanced $(Bu)_2$cAMP-stimulated testosterone production indicated that the action of HS is exerted, at least in part, at a point distal to the generation of cAMP.

Treatment of pig Leydig cells with exogenous pregnenolone, progesterone and 17α-hydroxyprogesterone augmented testosterone production, whereas HS significantly stimulated the conversion of 17α-hydroxyprogesterone to testosterone but did not affect the conversion of pregnenolone or progesterone to testosterone. These findings suggest that HS may specially enhance 17,20-desmolase activity without enhancing 17α-hydroxylase activity. Since both 17α-hydroxylase and 17,20-desmolase activities are found in microsomal cytochrome *P*-450, a single multifunctional enzyme complex, in pig testis [36] and no evidence is obtained in previous studies for the possible existence of a steroid 17α-hydroxylating *P*-450 without lyase activity [36,37], the significance of the selective enhancement by HS of 17,20-desmolase suggests additional investigation. Further studies using microsomal preparations are needed to examine this point. On the other hand, because a time study on the conversion of pregnenolone, progesterone or 17α-hydroxyprogesterone to their direct metabolites was not examined in the present investigation, the conclusive demonstration that HS can stimulate the activity of 17,20-desmolase needs further direct measurement of the enzyme activity in a cell-free system.

Our results also demonstrate that the stimulation of testosterone biosynthesis by HS was not associated with significant changes in total Leydig cell number or cellular protein content in these confluent cultures. These findings indicate that the stimulatory actions of HS on steroidogenesis are not due to an enhancement of cellular replication or survival. Loeb [38] also found that glucocorticoids had no effect on the incorporation of thymidine into the DNA of testicular tissue. In view of the general catabolic effect of glucocorticoid on proteins, one might suggest that the stimulation of testosterone production was due to a generalized effect on all protein synthesis. However, in our study, total cellular protein content of the Leydig cells was not changed by the HS treatment, suggesting that the increased steroidogenic response of Leydig cells to HS may be specific to hormone production. Since other intermediate steroids of the testosterone biosynthetic pathway were not measured, we do not know if the effect was specific for testosterone itself.

Mather *et al.* [39] and Haour *et al.* [40] have reported that both testosterone and dehydroepiandrosterone sulfate (DHAS) are significant secretory products of immature pig Leydig cells in culture. Moreover, the accumulation of

unconjugated steroids including testosterone is only linear during the first 4 h in culture, whereas conjugated steroids accumulate essentially linearly during the 36 h experimental period [41]. It appears that the measurement of testosterone alone after a long-term incubation, as in the present study, may not reflect the actual capacity of pig Leydig cells to produce testosterone. A modified experimental design for pig Leydig cell cultures will be required. One possibility is to incubate the cells first for 48 h with HS, then change the medium and measure testosterone production into the cell culture medium after a short-term incubation with or without LH, as previoulsy described [39–41]. Another possibility is to have a more complete profile of steroid products which should clearly include DHAS.

In the present investigation, porcine ACTH did not alter basal or LH-stimulated production of testosterone. These results suggest no direct involvement of ACTH in modulating testicular steroidogenesis. The results also lend support to the previous report of Schoen and Samuels [42], in which testicular androgen biosynthesis *in vitro* is not altered by ACTH.

In conclusion, the results of the present study indicate that HS can stimulate testosterone production by purified immature pig Leydig cells. The stimulatory effects of HS on cAMP production reported suggest that at least one primary site of HS action must be located at or before cAMP production. ACTH does not appear to directly modulate testosterone production.

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