Hypothalamic-pituitary disconnection in fetal sheep blocks the peripartum increases in adrenal responsiveness and adrenal ACTH receptor expression


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IN THE LATTER STAGES OF MAMMALIAN pregnancy, an increase in fetal plasma glucocorticoid concentration stimulates maturation of several organ systems, most notably the lung (5, 14). In sheep, where gestation is typically 147 days, this cortisol increase also initiates labor. It appears that increased pituitary secretion of ACTH and enhanced adrenal responsiveness both play important roles in mediating the prepartum increase in fetal plasma cortisol (5, 21). The enhanced adrenal responsiveness is also temporally linked to upregulation of ACTH receptor (ACTH-R) expression in the fetal adrenal (12, 16, 28). Interestingly, although it has been known for over a decade that hypothalamic-pituitary disconnection (HPD) abolishes the increase in fetal plasma cortisol, the precise mechanisms underlying this decrease remain unclear. We hypothesized that reductions in adrenal responsiveness and ACTH receptor (ACTH-R) expression may be mediating factors. HPD or sham surgery was performed at 120 days of gestation, and fetuses were placed in a latex glove contained by netting placed around the ewe's abdomen. Maternal and fetal arterial and venous polyvinyl catheters were inserted, exteriorized via a small incision in the maternal flank, and placed in a latex glove contained by netting placed around the ewe's abdomen.

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Blood sampling and necropsy. Fetal and maternal blood samples were obtained every second day for routine blood gas and pH determination using an ABL5 blood-gas analyzer (Radiometer, Copenhagen, Denmark). Aliquots of fetal plasma were also frozen at regular intervals for hormone analysis.

In late gestation (mean = 138 ± 0.4 dGA for HPD, 138 ± 0.1 dGA for sham), ewes were sedated with ketamine and anesthetized with halothane, and the fetuses were delivered by cesarean section. After this, both ewes and fetuses were killed by injection of pentobarbital sodium. Fetal adrenals were collected, and cortical tissue was isolated and snap-frozen or processed for cell culture. At this time, the completeness of HPD was verified visually.

Cell Dispersion and Culture

Adrenals from HPD and sham animals were cleaned, weighed, and bisected, and the medulla was gently peeled out. Cortical cells were dispersed in 0.4% collagenase, type I (Worthington Biochemical, Lakewood, NJ) for 2–3 h, washed, centrifuged through 60% Percoll (Sigma, St. Louis, MO), counted, diluted in DMEM-Ham’s F12–10% fetal calf serum, and plated in 48-well plates at 2 × 10^5 cells/well. Treatment paradigms were identical for both HPD and sham adrenals. After 48 h at 37°C in 5% CO2 atmosphere (day 1 of stimulation), the cells were rinsed two times with serum-free DMEM-Ham’s F12 medium containing 0.1% Polyep (Sigma). This medium was used for all subsequent treatments. After 1 h, the medium was removed and replaced with medium alone (unstimulated) or with ACTH-(1–24) (0.15 nM Cortrosyn; Organon, West Orange, NJ) for 2 h at which time the medium was removed and frozen for cortisol determination. [In our lab, 0.15 nM produces half-maximal cortisol secretion from ovine cells (24).] For the overnight incubation study (Table 1), wells were rinsed immediately two times, refilled with medium alone or with medium containing either 0.15 nM ACTH-(1–24) or 1 nM forskolin (FSK; Sigma), and incubated overnight (18–20 h). The next day (day 2 of stimulation), all cells were rinsed two times and stimulated with ACTH-(1–24) for 2 h. Again, the medium was removed and frozen. Cortisol secretion data for day 2 are included only when there were enough cells from an animal for a minimum of two wells for all three overnight treatments. Cells were then harvested for RNA extraction.

Cell Proliferation Assay

Adrenals from 120-dGA fetal sheep (n = 4) were dispersed as for cell culture. They were plated in triplicate in 96-well plates at 40,000 cells/well. To estimate cell proliferation that might take place in our paradigm, cells were treated as in Table 1 and the proliferation studies were done on day 2. Immediately after the final stimulation, the CellTiter 96 Aqueous One solution cell proliferation assay (item G3582, Promega, Madison, WI) was used to determine the number of viable cells. The optical density of each well was compared with a standard curve and regression analysis. Unknowns were quantitated using the standard curve and regression analysis. Data obtained were normalized to the 28S rRNA.

Cellular RNA extraction and competitive RT-PCR. The adrenal cortices, especially after HPD, yielded too few cells for measurement of mRNA by RPA. Therefore, we used an established quantitative RT-PCR assay for receptor mRNA quantitation from primary cultures of fetal adrenocortical cells. Total RNA was extracted from cells using an RNeasy kit (Qiagen, Hilden, Germany). Pellets of ~10^5 cells were suspended in 350 μl of lysis buffer [50 mM Tris·HCl (pH 8.0), 140 mM NaCl, 1.5 mM MgCl2, 0.5% Nonidet P-40], and the supernatant was loaded onto the column supplied in the kit. The concentration of RNA was determined by measuring absorbance at 260 nm, and the resultant RNA preparations were stored in aliquots at −80°C until use.

For more accurate measurement of mRNA levels, corrections for reaction to reaction variation in amplification efficiency are necessary. This is most commonly performed by inclusion of an internal control in the cDNA synthesis reaction. An internal standard is typically a synthetic or mutant RNA molecule containing the same primer template sequences as the experimental target. The internal standard is designed to generate a PCR product of a different size or to delete or add a restriction site to allow differentiation between the amplification productions of the target and the internal standard.

Competitive RT-PCR to determine ACTH-R mRNA levels was performed as previously described (6). ACTH-R primers were synthesized based on the sequence of the ovine ACTH-R cDNA (GenBank accession no. AF116874). The upstream primer was 5’-GGCTTTTTGCGAAAGAGATA-3’, and the downstream primer was 5’-GGCATGTGACGATGCTGTG-3’. Primers were designed such that the predicted sizes of PCR products were 360 and 143 bp for native and mutant ACTH-R, respectively. The mutant ACTH-R was identical to the native ACTH-R PCR product except for an engineered 217-bp deletion by restriction enzyme PstI. After linearization of the plasmids containing native and mutant ACTH-R cDNA, we synthesized corresponding ACTH-R cRNAs using an in vitro transcription system kit (Promega). Concentrations of native and mutant ACTH-R cRNA were determined by measuring absorbance at 260 nm.

For each RT reaction, either serially diluted native cRNA templates (31.25, 62.5, 125, 250, 500 fg) or 0.2 μg of total RNA from ovine adrenocortical cells, both with fixed amount of mutant cRNA template (25 fg), was denatured at 75°C for 3 min, cooled on ice, and then reverse transcribed to first-strand cDNA according to the manufacturer’s instruction (Promega) at 42°C for 60 min. The reaction was stopped by heating to 92°C for 10 min. PCR was carried out in triplicate in a total volume of 25 μl, containing 10× PCR buffer, dNTP mix, 10 μM of each corresponding primer, 1 U of thermostable DNA polymerase (Promega), and 1.0 μCi of [α-32P]dCTP (3,000 Ci/mmol). The reaction was started with an initial denaturation for 3 min at 94°C, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, followed by a final extension step of 10 min at 72°C. After PCR, 5 μl of each sample, including standard curve samples, were electrophoresed on 5% polyacrylamide gel. Gels were then exposed to x-ray film overnight, scanned by densitometry, Carlsbad, CA) according to the manufacturer’s recommendations. RNA was quantified by measuring absorbance at 260 nm and then stored in aliquots at −80°C until use.

We performed RNase protection assay (RPA) using an RPAII kit (Ambion, Austin, TX). Briefly, known quantities of [32P]-labeled antisense probe. After hybridization and incubation with RNP solution, the protected fragment from each reaction was precipitated and subsequently run on a 5% polyacrylamide gel containing 8 M urea. Gels were exposed to x-ray film overnight, and the amount of signal was detected by scanning laser densitometry. A standard curve was constructed by plotting standard RNA concentration vs. optical density of the signal from the protected fragment. Unknowns were quantitated using the standard curve and regression analysis. Data obtained were normalized to the 28S rRNA.

Table 1. Cell culture experimental design

<table>
<thead>
<tr>
<th>Day 1 of Stimulation (Fig. 6), 3rd Day of Culture</th>
<th>Overnight Treatment</th>
<th>Day 2 of Stimulation (Fig. 7)</th>
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<tr>
<td>2-h ACTH stimulation Medium or ACTH or FSK</td>
<td>2-h ACTH stimulation</td>
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Treatment was the same for both sham and hypothalamic-pituitary disconnection (HPD) cells. All cells were stimulated on day 1 and day 2 with ACTH. Overnight incubation was in medium alone or with ACTH or forskolin (FSK).
and analyzed with TINA software (version 2.09, Raytest). The amount of signal was quantified with the regression equation from the standard curve of native ACTH-R. Data were converted from optical density readings to femtogram of mRNA per microgram of total RNA and reported as ratios of each unknown sample against mutant signals.

**Western blot for ACTH-R protein.** Protein extraction for Western blot analysis was done according to previously described methods (26). Equal amounts of protein were loaded onto 12% SDS-polyacrylamide gels, subjected to electrophoresis, and then transferred to PVDF membranes. Nonspecific binding was blocked with 5% nonfat dried milk in TWEEN 20-Tris-buffered saline before incubation at 4°C overnight with rabbit polyclonal antibody to amino acids (87-101) of ovine ACTH-R protein (26) and then to sheep anti-rabbit IgG linked to a horseradish peroxidase. The enhanced chemiluminescent (Amer- sham Pharmacia Biotech, Piscataway, NJ) blots were visualized on film and quantified by scanning densitometry. Results are reported in arbitrary optical density units.

**Hormone Measurement**

Cortisol was measured by radioimmunoassay using a kit from Diagnostic Systems Laboratories (DSL2000, Webster, TX) that measures total cortisol. This assay has been validated for measuring ovine fetal cortisol levels. The minimum detectable amount of cortisol was 0.6 ng/ml. Coefficients of variation were 4.2% intra-assay and 7.0% interassay.

ACTH-like bioactivity was defined as the ability of ACTH extracted from plasma to cause secretion of cortisol from dispersed adult ovine adrenal cortical cells compared with the cortisol secreted in response to known quantities of ACTH-(1–24). Details of the plasma extraction and bioassay have been published (4, 30). Briefly, 1 ml of each plasma sample was added to 70 mg of 100 mesh glass and then washed with 0.05 M phosphate buffer. ACTH-like activity was eluted from the glass with 0.25 N HCl-acetone (60:40). The solvent was dried in a SpeedVAC (Savant Instruments, Farmingdale, NY) and subjected to electrophoresis before incubation at 4°C overnight with rabbit polyclonal antibody to amino acids (87-101) of ovine ACTH-R protein (26) and then to sheep anti-rabbit IgG linked to a horseradish peroxidase. The enhanced chemiluminescent (Amer- sham Pharmacia Biotech, Piscataway, NJ) blots were visualized on film and quantified by scanning densitometry. Results are reported in arbitrary optical density units.

**Plasma Cortisol**

Mean plasma cortisol levels at 122–126 dGA were similar in sham (4.4 ± 0.4 ng/ml) and HPD (3.8 ± 0.6 ng/ml) groups. By 138 dGA, values were 20-fold greater in sham than in HPD animals (45.2 ± 18.9 ng/ml in sham vs. 2.5 ± 1.9 ng/ml in HPD; \( F = 13.1, P < 0.01 \)).

**ACTH-like Bioactivity**

Mean plasma ACTH-like bioactivity at 120–129 dGA was similar for both HPD and sham groups. By 135–139 dGA, ACTH bioactivity had increased (\( F = 8.4, P < 0.01 \)) in the sham group, whereas ACTH bioactivity did not change during this time in HPD animals (\( F = 0.15, P > 0.05 \)).

**Statistical Analysis**

Data were compared using ANOVA or \( t \)-test. Where appropriate, post hoc differences were determined with the Newman-Keuls test. All data are presented as means ± SE, and differences were considered to be significant at \( P < 0.05 \).

**RESULTS**

**Animal Data**

HPD was verified at necropsy by visual examination of the lesion and pituitary. We have shown that anterior pituitaries from HPD animals are functional by in vitro studies (29).

On the day of necropsy, the body weights of both HPD and sham animals were the same (3.7 ± 0.2 and 3.4 ± 0.3 kg, respectively), whereas the mean adrenal weight (wet weight of both adrenals) of the HPD group was significantly less than the weight for the sham group (294.6 ± 23.8 vs. 464.8 ± 36.2 mg) (Fig. 1). The HPD group weight is comparable to that of adrenals from unoperated fetuses at 120 dGA (229.5 ± 12.8 mg; \( n = 13 \)). There was a 50% reduction in the ratio of adrenal weight (mg) to body weight (kg) in the HPD group (73.2 ± 5.4 mg HPD vs. 146.1 ± 17.3 mg sham; \( P < 0.01 \)).

The indicators of fetal health were normal in both groups and did not change over the duration of the study period. There were no between-group differences for pH (7.353 ± 0.006), \( P_{O2} \) (21.2 ± 0.3 Torr), \( P_{CO2} \) (50.7 ± 1.4 Torr), or hematocrit (30.3 ± 0.8%).

Mean plasma cortisol levels at 122–126 dGA were similar in sham (4.4 ± 0.4 ng/ml) and HPD (3.8 ± 0.6 ng/ml) groups. By 138 dGA, values were 20-fold greater in sham than in HPD animals (45.2 ± 18.9 ng/ml in sham vs. 2.5 ± 1.9 ng/ml in HPD; \( F = 13.1, P < 0.01 \)).
Adrenal ACTH-R mRNA Levels

RPA performed on the extracted adrenal cortices of sham and HPD fetuses indicated that ACTH-R mRNA levels were 50% lower in tissue from the HPD group (9.8 ± 1 pg/10 μg of total RNA sham vs. 4.1 ± 0.5 pg/10 μg of total RNA HPD). The ratio of ACTH mRNA to 28S rRNA was also significantly lower in the HPD group (Fig. 3).

Adrenal ACTH-R Protein Levels

The level of receptor protein expression was significantly lower (37%) in adrenals from HPD fetuses (Fig. 4).

ACTH-R mRNA Levels in Adrenal Cortical Cells

To measure ACTH-R mRNA in the same cells used for the cortisol secretion studies, competitive RT-PCR was performed. For more accurate results, a mutant ACTH-R RNA was synthesized and included in each reaction. The amount of mRNA of each sample is expressed as a ratio to that of mutant mRNA. ACTH-R mRNA expression in adrenocortical cells from HPD animals was approximately one-third of that in cells from sham animals after overnight incubation with medium alone. Overnight incubation with ACTH or FSK increased ACTH-R mRNA levels approximately twofold in HPD animals (treatment effect; \( F = 7.9, P = 0.01 \)). Likewise, overnight incubation with ACTH or FSK increased receptor mRNA expression in sham cells (treatment effect; \( F = 76.0, P < 0.02 \)), but the change represented an ~40% increase (\( P < 0.05 \) vs. HPD). In all instances, receptor mRNA levels were higher in adrenal cells from sham animals (group effect; \( F = 46.3, P < 0.01 \)) (Fig. 5).

Cell Secretion and Proliferation Data

HPD reduced both basal and ACTH-stimulated cortisol secretion by primary cultures of adrenocortical cells on day 1 (group effect; \( F = 248.3, P < 0.01 \)). On the first day of study, basal cortisol secretion was barely detectable from HPD or sham cells (Fig. 6). Moreover, there was no significant cortisol response to ACTH by HPD cells (treatment effect; \( F = 0.8, P > 0.05 \)), whereas secretion was increased over 50-fold in sham cells (treatment effect; \( F = 717.8, P < 0.01 \)) (Fig. 6).

Overnight exposure of cells from the HPD animals to either ACTH or FSK enhanced the cortisol responses to stimulation (treatment effect; \( F = 32.5, P < 0.01 \)). Indeed, the responses to ACTH by HPD cells on day 2 (Fig. 7) after overnight incubation with the secretagogues were not different from the cortisol responses to ACTH in the sham cells on day 1 (Fig. 6). Similarly, overnight incubation with ACTH or FSK increased ACTH-induced cortisol secretion by sham cells compared with responses after overnight incubation with medium alone (treatment effect; \( F = 50.4, P < 0.01 \)). The cortisol secretion on day 2 by the sham cells was significantly greater than that from HPD cells on day 2 (Fig. 6) for each incubation paradigm (group effect; \( F = 9.8, P = 0.01 \)). Overnight incubation of cells from either group in medium alone did not significantly change cortisol responses to ACTH on day 2 compared with day 1 (group effect; \( F = 1.3, P > 0.05 \)) (Figs. 6 and 7).

There was no effect of overnight incubation with ACTH or FSK on adrenocortical cell number in 120-dGA cells from unoperated animals.

DISCUSSION

The goal of this study was to determine a possible explanation for the observation that HPD in fetal sheep blocks the rise in fetal plasma cortisol that normally occurs in late gestation. We hypothesized that this may be related to attenuation of the increase in adrenal responsiveness to ACTH that also occurs close to term in this species. Our results demonstrate that this is the case; i.e., adrenal cortical cells obtained from fetuses subjected to HPD show minimal steroidogenic responses to a single direct exposure to ACTH, whereas cells from sham-operated animals increase cortisol secretion 50-fold. Moreover, our data suggest that this loss in adrenal responsiveness is related to HPD-induced blockade of the developmental increase in adrenal ACTH-R expression normally occurring in late gestation. These changes may be the result of impaired secretion of bioactive ACTH in late gestation in the fetuses that have the connection between the hypothalamus and pituitary disrupted.

We used a sensitive bioassay to assess tropic drive to the adrenal in HPD and control fetuses. We found that HPD blocked the increase in bioactive ACTH that normally occurs in late gestation (4). Plasma bioactive ACTH increased almost threefold in sham fetuses, between 120 and 138 dGA, whereas HPD fetuses maintained the 120-dGA bioactive ACTH concentration. These results are similar to those of Phillips et al. (19), who used an immunoradiometric assay to measure...
ACTH, but are counter to the report of Deayton et al. (10). The reduction in bioactive ACTH was associated with significantly lower ACTH-R mRNA and ACTH-R protein levels in the fetal adrenal, which suggests a coupling between tropic input to the adrenal in vivo and ACTH-R expression. Additional observations that support a link between plasma ACTH and fetal adrenal ACTH-R expression in vivo are the strong positive linear relationship between fetal plasma ACTH-(1–39) levels and ACTH-R mRNA in the fetal adrenal, the fact that infusion of cortisol into the fetus suppresses both ACTH-(1–39) and ACTH-R mRNA (28), and the report that hypoxia in late gestation increases ACTH-R mRNA in the fetal adrenal (12). Admittedly, the positive correlation between plasma ACTH and cortisol complicates the separation of the effects of the two on ACTH-R expression. This study was designed to document the effects of HPD on ACTH-R expression and adrenal responsiveness.

We also found that adrenal cortical cells isolated from HPD animals were unresponsive to ACTH stimulation with regard to cortisol secretion. However, overnight incubation with ACTH restored cortisol synthetic ability to levels seen in adrenal cells from sham animals and reversed the ACTH-R mRNA deficit in the HPD cells. This restoration of receptor mRNA levels and steroidogenic capacity by ACTH suggests that 1) adrenal ACTH-R reduction has a physiological consequence in vivo and 2) the impact of reduced tonic stimulation of the adrenal in vivo can be reversed by incubation with ACTH in vitro. A positive effect of ACTH on ACTH-R mRNA levels in normal adrenal cells in vitro has also been reported (15, 17, 18) and is consistent with our findings.

Considering the above, it is surprising that there are two reports that ACTH-(1–24) infusions in fetal sheep do not change ACTH-R mRNA levels (3, 25). This may be due to changes in mRNA levels below the assay detection level (25) or doses of ACTH below the threshold for producing an effect on receptor expression.

We have previously shown that both ACTH-R binding capacity (number of receptors per cell) and ACTH-R mRNA levels increase proportionately over the last third of gestation (28), thereby indicating that changes in ACTH-R mRNA reflect changes in receptor expression in the fetal adrenal. In this study, we demonstrated that HPD produces parallel and similar decreases in ACTH-R mRNA and protein as measured by Western blot. These data also support the concept that, at least in these experiments, changes in ACTH-R mRNA are indicative of changes in adrenal receptor expression.
It is interesting that FSK, an agent that directly stimulates the production of cAMP, is also effective in restoring the ACTH-R and steroidogenic responses to the peptide. The effects of ACTH on its receptor mRNA are thought to be via a cAMP pathway (17), and thus the mediator for the effect of both agents could be cAMP. In addition, because both agents increased cortisol production, the steroid could be responsible for the stimulation observed. Dexamethasone has been shown to increase ACTH-R mRNA (20) as well as receptor binding of ACTH (9) in vitro in adult ovine adrenal cells. Support for a role for cAMP in increasing ACTH-R expression is found in studies of JEG3 and Cos1 cells cotransfected with steroidogenic factor-1 and the 5′ regulatory region of the ACTH-R gene. In these nonadrenal cells, ACTH-R gene promoter activity is enhanced by FSK treatment (23). The relative importance of cAMP and cortisol in affecting ACTH-R expression is unclear at present.

Developmentally, the 120-dGA fetal adrenal is relatively quiescent. Between 130 and 144 dGA there is a period of cellular hypertrophy and hyperplasia (2). Although ACTH is generally regarded as a primary regulator of this development, ACTH is not mitogenic in primary cultures of adult bovine (13) or human fetal (11) adrenocortical cells. We evaluated the effect of ACTH or FSK on the number of viable cells from 120-dGA ovine fetuses and found that fetal adrenal cortical cell number was not increased by overnight incubation with ACTH or FSK. Although this does not totally eliminate hyperplasia as an explanation for increased cell responsiveness in the HPD cells, with respect to both weight and ACTH responsiveness in vitro, the HPD adrenal closely resembles that of the 120-dGA adrenal (27). Thus it seems unlikely that the ACTH-induced increases in receptor expression can be attributed to changes in cell number.

The significant difference in adrenal weight between the HPD and the sham fetuses at 138 dGA in the present study strongly implies that an intact hypothalamic-pituitary connection is essential for adrenal cortical development. In fact, the HPD adrenals were comparable in size to those from unoperated 120-dGA fetuses. The association of reduced adrenal weight and reduced adrenal weight-to-body weight ratios with lower plasma levels of bioactive ACTH is consistent with the importance of ACTH for fetal adrenal growth. Recently, Coll...
et al. (7) demonstrated that exposure to ACTH alone can restore morphology and functionality to significantly atrophic and hypofunctional adrenals from proopiomelanocortin-deficient mice. The fact that adrenal weight can be partially restored and adrenal weight-to-body weight ratios fully restored by infusion of ACTH-(1–24) into animals subjected to HPD (20) further supports the possibility that the adrenal growth impairment that we observed is related to the reduced circulating levels of bioactive ACTH. Although there is not uniform agreement on the effects of HPD on fetal adrenal weight, our results are consistent with reports that HPD at 106–120 dGA diminishes adrenal growth during the remainder of gestation (8, 19, 22).

In summary, we have shown that HPD in the fetus blocks the prepartum increase in bioactive ACTH, adrenal ACTH-R expression, and adrenal responsiveness to ACTH. It is likely that these changes explain, at least in part, the absence of the elevation in fetal plasma cortisol normally found in late gestation. Although the adrenal cortical cells from the HPD fetuses are initially unresponsive to ACTH, both ACTH-R expression and cortisol secretory ability are restored by ACTH. These findings support the premise that HPD and the subsequent reduction in biologically active ACTH prevent the normal development of ACTH-R expression and adrenal responsiveness. In addition, the suspension of adrenal cortical functional development in vivo and subsequent restoration by

![Fig. 6](image-url)  
**Fig. 6.** Cortisol secretion from adrenal cortical cells on day 1. *A*: no stimulation; *B*: ACTH stimulation (n = 6 for sham, n = 5 for HPD). Different letters indicate significant differences between groups.

![Fig. 7](image-url)  
**Fig. 7.** Cortisol secretion from sham (*A*) and HPD (*B*) adrenal cortical cells in response to ACTH stimulation on day 2. Cells were stimulated with ACTH on day 1, incubated overnight with medium, ACTH, or FSK, and then stimulated again with ACTH (n = 6 for sham; n = 5 for HPD). Both group and treatment effects are present. For each treatment, HPD cells secreted less cortisol than sham cells. In both sham and HPD groups, treatment with ACTH or FSK significantly increased cortisol secretion. Different letters indicate significant differences between groups.
ACTH in vitro emphasizes the importance of the peptide for the maturation of the adrenal cortex.

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GRANTS

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