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The Impact of ACTH Receptor Knockdown on Fetal and Adult Ovine Adrenocortical Cell Function

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Abstract

Preparing the mammalian fetus for birth requires an increase in fetal plasma glucocorticoid levels. The mechanisms facilitating this increase are not fully known. It has been shown in sheep that the prepartum elevation in fetal plasma cortisol is accompanied by increases in adrenocorticotropin receptor (ACTH-R) expression in the fetal adrenal and in the adrenal responsiveness to stimulation. To determine the significance of the upregulation in ACTH-R expression on fetal adrenal function, the authors used small interfering RNA targeted to the ovine ACTH-R to reduce receptor expression and studied responses to stimulation in ovine adrenal cells. They studied fetal cells from late gestation after responsiveness had increased. They also studied adult cells to determine if maturation would influence the impact of receptor expression suppression on responsiveness. Fetal and adult cells were obtained, dispersed, transfected with receptor-targeted small interfering RNA or scrambled small interfering RNA, and subsequently stimulated with ACTH. Cells and media were harvested for measurements of gene and protein expression and cyclic adenosine monophosphate (cAMP) and cortisol levels. The ability of ACTH to upregulate its receptor or steroid acute regulatory protein was attenuated in fetal ($P < .01$) and adult cells ($P < .01$) by small interfering RNA treatment; the blockade was more pronounced in the adult cells ($P < .01$). The small interfering RNA treatment also blocked the cAMP response to ACTH in fetal ($P < .001$) and adult ($P < .05$) cells. This was accompanied by marked reductions in cortisol responses in both ($P < .001$ and $P < .01$, respectively). These data suggest that upregulation of the ACTH-R expression in late gestation is essential for the increase in adrenal steroidogenic capacity occurring then. The data also indicate that a reduction in the ACTH-R expression blocks the ability of the peptide to stimulate early steps in the steroidogenic pathway event after maturation is complete.

Keywords

small interfering RNA; adrenal gland; fetal

A late-gestation increase in fetal glucocorticoid concentration is a hallmark of fetal development in mammalian species and plays a critical role in preparing the fetus for the transition to extrauterine life. In particular, there appears to be an absolute requirement for an elevation in fetal plasma cortisol to promote lung maturation prior to birth. In addition, for a number of species, including the sheep, the increase in fetal cortisol production is also the critical trigger determining the timing of labor.¹

Adrenocorticotropin (ACTH) has been demonstrated as the major regulator of cortisol production as well as of the expression of key genes essential for cortisol biosynthesis in the

fetal adrenal. ACTH acts via a 7-transmembrane domain receptor belonging to the G protein-coupled receptor superfamily, leading to activation of the adenylyl cyclase pathway and subsequently protein kinase A.² The ACTH receptor (ACTH-R; melanocortin 2 receptor) is mainly expressed in the adrenal cortex. Substantial progress in understanding how ACTH-R expression is regulated has been made since the cloning of the melanocortin 2 receptor gene by Cone and colleagues in 1992.³

In vitro studies have shown a positive effect of ACTH on its receptor expression in both fetal and adult adrenal cells,⁴⁻⁷ and during development, ACTH-R expression increases in the fetal adrenal.^{8,9} This increase is accompanied by an increase in adrenal steroidogenic responsiveness.¹⁰⁻¹³ Nevertheless, the significance of the upregulation of ACTH-R expression and the increase in steroidogenic capacity has not been firmly established.

There are also changes in steroid acute regulatory protein (StAR) expression in the fetal adrenal, which are qualitatively similar to the changes in the ACTH-R.¹⁴ These changes appear to be important for maturation of steroidogenic capacity because StAR protein mediates the rate-limiting and acutely regulated step in steroidogenesis, the transfer of cholesterol from the outer to the inner mitochondrial membrane, where the cytochrome P450 side chain cleavage (P450_{sc}) enzyme initiates the synthesis of all steroid hormones.¹⁵

In the present study, we determine (1) if ACTH-R expression upregulation in the fetal adrenal in late gestation is required for the increased cortisol response to ACTH seen at this time and (2) if reducing the ACTH-R expression in adult adrenal cells with a mature complement of all components of the cortisol biosynthetic pathway has a physiological impact. To answer these questions, we use RNA interference to suppress the expression of the adrenal ACTH-R. RNA interference is the process of gene silencing in which the recognition of double-stranded RNA ultimately leads to posttranscriptional suppression of gene expression.¹⁶⁻¹⁸ The agent responsible is generally called small interfering RNA (siRNA).¹⁹ We hypothesize that siRNA treatment targeted to the ACTH-R on the fetal adrenal will block the enhanced responsiveness to ACTH seen in late gestation and that this will be related to blocking the upregulation of StAR in the adrenal and the generation of cyclic adenosine monophosphate (cAMP) by adenylyl cyclase.

MATERIALS AND METHODS

Preparation of Small Interfering RNA

Gene silencing was performed according to a plasmid-based siRNA method. Plasmid vectors encoding siRNA targeted to the ACTH receptor were constructed as follows. Two oligonucleotides consisting of a sense fragment and an antisense fragment were annealed in vitro, and the resultant double-stranded DNA fragments were sub-cloned into the BamHI-HindIII site of pRNATin-H1.2/Neo vector (Genscript, Piscataway, NJ). The siRNA sequences targeting ovine ACTH-R corresponded to the coding regions +908 to +928 (CCAGGTGAATGGTGTGTTGAT). All constructs were verified by DNA sequencing. The vector also contained green fluorescent protein (GFP) to monitor transfection efficiency.

Cell Culture and Transfection

All procedures using animals were approved by the institutional Animal Care and Use Committee. Pregnant ewes between 135 and 138 days of gestation were brought to the laboratory at least 2 days prior to obtaining adrenals. On the day of tissue harvest, the ewes were sedated with ketamine and anesthetized with isoflurane, and the fetuses were delivered by cesarean section. The fetuses (N = 9) were euthanized with an overdose of pentobarbital, and the adrenals were rapidly obtained using aseptic technique and placed in sterile ice-cold

media. The adult (N = 5) adrenals were obtained in a similar fashion. Adrenal cortex was separated from medullary tissue by blunt dissection, the cortical tissue was dispersed, and adrenocortical cells (ACs) were cultured as previously described.²⁰ After 3 days in culture, the AC cells were transfected with siRNA expression vector or control vector with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The vector containing a scrambled siRNA sequence was used as a negative control. Cells were transfected at 90% to 95% confluency. At 38 hours, cells were exposed to ACTH₁₋₂₄ (1.5×10^{-10} M), and after further incubation, media were collected and cells were harvested at 48 or 72 hours (for mRNA and protein measurements, respectively) after transfection. The supernatant was stored at -20°C until assayed for cortisol or cAMP concentrations.

Cell Viability Assay

To study the effects of siRNA treatment on cell viability, adult cells were cultured as described and transfected with ACTH-R siRNA vector or scrambled siRNA vector (control). After a 48-hour incubation, viable cells were quantified by counting after harvesting the cells with trypsin digestion and adding an equivalent volume of 0.4% trypan blue solution to an aliquot of resuspended cells. The stained and unstained cells were counted by means of a hemacytometer.

RNA Isolation and RNase Protection Assays

Cultured cells were lysed in buffer containing β -mercaptoethanol and processed for RNA extraction using the RNeasy column (Qiagen, Valencia, CA). Amounts of RNA were quantitated by measuring absorbency at 260/280 nm; ACTH-R and StAR mRNA levels were measured by RNase protection assays (RPAII Kit; Ambion, Austin, TX) as described previously.⁶ Briefly, plasmids containing cDNAs for ovine ACTH-R, StAR, and 28S rRNA were linearized with EcoR I, Hind III, and Hind III, respectively. Then, ³²P-labeled antisense riboprobes were generated for hybridization. The protected fragments were fractionated by electrophoresis through a 5% denaturing polyacrylamide gel containing 8 M urea. After electrophoresis, the gel was exposed to Kodak medical x-ray film. The relative level of mRNA for ACTH-R or StAR was calculated by dividing the band intensity of a given mRNA by the band intensity of the corresponding 28S rRNA in each sample. Frozen aliquots of an RNA pool were used as an internal control. One aliquot of RNA pool was measured in each experiment to correct for interassay variations. The interassay coefficients of variation for ACTH-R and StAR mRNA were 8.15% and 8.66%, respectively.

Western Blot Analysis

Cells were lysed, and the lysate was incubated on ice for 30 minutes. Protein concentration was determined by bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL). Equal amounts (50 μ g) of protein were resolved on 12% polyacrylamide gels (Bio-Rad) and blotted onto Immobilon-P polyvinylidene difluoride membranes. Western blotting was carried out as described previously⁶ using polyclonal anti-ovine ACTH-R antisera (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-mouse StAR antibody (Affinity Bioreagents, Golden, CO). After blocking with 5% nonfat dry milk, membranes were incubated with appropriate antibody overnight at 4°C, washed 3 times with Tris-buffered saline with 0.05% Tween 20 (TTBS), incubated with conjugated secondary antibody (1:10 000) for 1 hour, and washed 3 times with TTBS; then, the signal was visualized by chemiluminescence using an ECL reagent (Amersham Biosciences, Piscataway, NJ). Band intensities were quantified by densitometric scanning, and the results are reported in arbitrary optical density units. Lysates from control and siRNA-treated cells were run together in all experiments, and a molecular weight marker was included. Negative controls (data not shown) used nonimmune sera in place of antiserum for the proteins.⁶

Cortisol Assay

The concentration of cortisol in the medium was measured by immunoassay using an radioimmunoassay kit from Diagnostic Systems Laboratories (Webster, TX) according to the manufacturer's instructions. The cross-reactivity of the antibody presents 100% with cortisol, 33% with prednisolone, 9.3% with corticosterone, and 3.8% with 11-deoxycortisol. The assay sensitivity and intra-assay and interassay coefficients of variation were 0.6 ng/mL, 4.2%, and 7.0%, respectively.

cAMP Assay

The concentration of cAMP level in the medium was determined by enzyme-linked immunoassay using a kit from Cayman Clinical Company (Ann Arbor, MI) according to the manufacturer's instructions. The cross-reactivity of the antibody presents 100% with cAMP and 1.5% with cyclic guanosine monophosphate. The assay sensitivity and intra-assay and interassay coefficients of variation were 3 pmol/mL, 5.9%, and 9.3%, respectively.

Transfection Efficiency

Vector containing GFP was used to determine transfection efficiency. The number of cells containing GFP was counted in 3 replicate fields using fluorescence microscopy and expressed as a percentage of the total number of cells present. This was done in 3 separate experiments for both adult and fetal cells.

Statistics

All the experiments were performed with AC cells from 5 to 9 animals. All values are expressed as the mean $\pm$ SEM. Statistical analysis was performed using analyses of variance with Neuman-Keuls as the post hoc test. A P value $<.05$ was considered significant or t test where appropriate.

RESULTS

Transfected cells were visualized using fluorescence microscopy, and transfection efficiency was determined 48 hours after transfection. The rate of transfection ranged from 25% to 30%. Figure 1 shows typical light and fluorescence fields of transfected (adult) cells.

To determine whether reduction of mRNA and protein level correlated to cell death, cell viability was assessed by exclusion of trypan blue. Adrenal cell viability after dispersion but before aliquoting cells in wells is routinely 90% or higher in our laboratories. A slightly reduced cell viability was observed at the end of these experiments after the cells were lifted from the wells. However, there was no significant difference in siRNA-treated cells as compared (by t test) with the scramble vector-treated cells, as shown in Table 1.

Fetal Studies

As shown in Figure 2, ACTH could upregulate its receptor mRNA expression in only control vector-transfected cells ($F = 16.9$, $P < .001$).

Transfection with siRNA for 48 hours significantly reduced StAR mRNA in fetal AC cells and abolished the response to ACTH ($F = 14.8$, $P < .001$; Figure 3).

There was an increase in cAMP concentration after ACTH stimulation of the fetal AC cells ($F = 31.8$, $P < .001$), and the response was significantly attenuated in the siRNA-treated cells (Figure 4A).

ACTH receptor knockdown in fetal AC cells significantly decreased the cortisol response to ACTH ($F = 11.2$, $P < .00$; Figure 4B).

Adult Studies

The response of ACTH-R mRNA to stimulation in adult cells was suppressed by siRNA treatment ($F = 25.6$, $P = .01$; Figure 5A).

Immunoblot analysis of cell extracts was carried out to determine whether decreased receptor mRNA expression, as described above, correlated with decreased translation of the receptor protein. A similar trend was observed (Figure 5B). That is, ACTH-R protein was reduced after siRNA treatment and increased only after ACTH treatment in the control vector-treated cells ($F = 18.8$, $P < .0001$).

Knockdown of ACTH-R decreased in the StAR mRNA response to ACTH stimulation ($F = 49.4$, $P < .0001$; Figure 6A).

There was a significant inhibitory effect on StAR protein expression ($F = 36.4$, $P < .0001$; Figure 6B), and the response to ACTH did not exceed control values in the siRNA-treated cells.

The ability of ACTH to increase cAMP levels in the media was confined to vector-transfected cells ($F = 14.3$, $P < .0003$; Figure 7A).

Similarly, the siRNA-mediated reduction of ACTH-R expression was accompanied by a decrease in cortisol secretion in response to ACTH ($F = 48.2$, $P < .0001$; Figure 7B).

Fetal Versus Adult Comparison

The overall levels of ACTH-R mRNA and StAR mRNA were similar in the fetal and adult animals. However, in responses to stimulation, there was an ACTH $\times$ Group interaction with mRNA levels for the ACTH receptor ($F = 7.79$, $P < .01$), increasing more in the adult animals. There was a trend for this to be the case with StAR mRNA as well ($F = 3.9$, $P = .07$). When the responses to ACTH in the control vector-treated cells were compared at the 2 ages, the percentage increases for both the ACTH mRNA (148.6 ± 33.2 vs 33.2 ± 8.7 , $P < .01$) and the StAR mRNA (348.0 ± 62.9 vs 51.4 ± 12.2 , $P < .01$) were greater in the adult cells. When cAMP levels were compared between ages, there was a marked group difference, with levels from adult cells being greater than levels from fetal cells ($F = 60.8$, $P < .0001$). There was an ACTH $\times$ Group interaction ($F = 57.6$, $P < .0001$), with higher levels in the adult cells. Likewise, the percentage increase of cAMP in control adult cells after ACTH was greater than that observed in the fetal cells (8229.4 ± 2806.0 vs 1266.7 ± 220.5 , $P < .01$). The cortisol levels were also different in the 2 groups ($F = 77.2$, $P < .0001$), and there was an ACTH $\times$ Group interaction ($F = 80.9$, $P < .0001$). The percentage increase in cortisol release by the adult control cells following ACTH stimulation tended to be greater than that from the fetal cells (2072.6 ± 642.0 vs 1082.2 ± 412.2 , $P = .10$).

The ability of the siRNA to suppress mRNA responses to ACTH was also different between the groups. There was significantly greater suppression of both the ACTH-R mRNA and StAR mRNA response to stimulation in the adult cells. In contrast, the suppression of the cAMP and cortisol responses to ACTH was similar (Figure 8).

DISCUSSION

One goal of the present study was to determine the importance of the upregulation of the fetal adrenal ACTH-R in late gestation on the enhanced responsiveness to ACTH that occurs then.

To achieve this, we compared responses to stimulation in fetal adrenal cells obtained close to term and transfected with either vector or siRNA targeted to reduce expression of the ACTH receptor. Another goal was to ascertain if, in adult cells where the entire steroidogenic pathway has matured, partial suppression of ACTH-R expression would have a negative impact on responses to stimulation comparable with that observed in fetal cells. Our data show that suppression of ACTH-R expression in late gestation, when the fetal adrenal has heightened receptor levels and steroidogenic capacity, abolishes cortisol responses to stimulation in vitro. Moreover, in adult cells in which the entire steroidogenic pathway is mature, the ability of ACTH to enhance cortisol production is blocked by partial knockdown of ACTH-Rs with ACTH-R siRNA. Our results suggest that the effects on cortisol production are related to attenuation of the initial steps in the steroidogenic cascade. Thus, it appears that enhanced ACTH-R expression close to term is necessary for the normal developmental increase in cortisol secretory capacity occurring then and for the maintenance of adrenal responsiveness in adult cells.

In all mammalian species in which it has been studied, there is an increase in fetal plasma glucocorticoid concentration at the end of gestation, which prepares the fetus for extrauterine life. A number of factors are thought to contribute to the increased cortisol concentrations in late gestation. It is clear that an intact hypothalamo-pituitary-adrenal axis is crucial for the normal increments in cortisol, as disruption at any level of the axis results in decreased cortisol concentrations.²⁰⁻²³ The available evidence indicates that an elevation in bioactive ACTH is a prerequisite for the elevation in cortisol and that the adrenal becomes more responsive to ACTH in late gestation.^{7,11,13} While the mechanisms responsible for the increase in adrenal responsiveness are not fully established, some data support a role for increased ACTH-R expression as being causative. For example, the increase in responsiveness is accompanied by an increase in ACTH-R expression, while the hypothalamic-pituitary disconnection (HPD) that blocks the upregulation of receptors in late gestation results in low fetal plasma cortisol levels and loss of adrenal responsiveness.⁷ Moreover, when adrenal cells from HPD animals are incubated with ACTH for 18 to 20 hours, ACTH-R expression and responsiveness are restored.⁷ In addition, although there is no uniform agreement,^{24,25} infusion of ACTH at 2 different fetal ages has been shown to upregulate the ACTH-R and increase adrenal responsiveness.¹⁰ These data are consistent in that they link alterations in ACTH-R expression with parallel changes in adrenal responsiveness.

The data in the current study strongly support the idea that the late gestation increase in adrenal responsiveness is mediated by increases in ACTH-R expression since knockdown of receptor levels causes loss of cortisol responses to ACTH in fetal adrenal cells obtained close to term. Indeed, the physiological importance of the upregulation of the ACTH-R in adrenal cells in late-gestation fetuses is found by comparison of cortisol secretion from those cells with cortisol secretion from adrenal cells obtained at 120 days of gestation. When stimulated with the same dose of ACTH, cells from 120-day-old fetuses secrete 15.0 ± 2.9 ng/mL (N = 9),²⁶ while the siRNA-transfected cells in the present study secreted 14.3 ± 5.1 ng/mL (N = 9). Thus, knockdown of the upregulated ACTH-R in late-gestation fetal adrenal cells reduces their ability to secrete cortisol to that seen at 120 days of gestation, when the fetal adrenal is relatively insensitive to ACTH stimulation.

We found similar results in adult cells, which have a fully mature steroidogenic pathway and higher levels of ACTH-R, StAR, and the enzymes involved in cortisol synthesis relative to the fetus at 0.6 to 0.9 gestation.^{9,14,27-29} This emphasizes how important the maturational increase in receptor expression is for ACTH-induced cortisol secretion even in the adult. Results of studies of inherited ACTH insensitivity also point to the ACTH-R as crucial for maintaining normal steroid secretion.³⁰ Indeed, a 1-base exchange in the ACTH-R promoter results in impaired cortisol responses to prolonged infusions of ACTH. Thus, relatively minor

alterations in the ACTH-R gene are clearly associated with attenuated cortisol responses to the secretagogue in humans.³¹

A major effect of the knockdown of the ACTH-R was blockade of the earliest steps in the steroidogenic pathway. That is, receptor knockdown compromises the ability of ACTH to increase cAMP generation and StAR expression. StAR is the key mediator in the delivery of cholesterol from the outer to the inner mitochondrial brain, where it can be acted upon by the first enzyme in the steroid biosynthetic pathway, cholesterol side chain cleavage enzyme (P450_{scc}). The ability of ACTH to increase StAR expression was blocked in both fetal and adult adrenal cells by the siRNA treatment. We have shown that StAR is upregulated by ACTH in fetal adrenal cells in vitro⁶ and that ACTH infusions in fetal lambs increase StAR expression.¹⁰ Others have reported that ACTH increases expression of StAR in adult bovine and human adrenal cortical cells in vivo and in vitro.^{4,29} Our data showing stimulation in control vector-treated cells are consistent with these observations. It is likely that the lack of increase in StAR in the present study was the result of blockade of the normal cAMP response to ACTH. There is substantial evidence indicating that cAMP works through a protein kinase A-mediated mechanism to regulate the expression of StAR in the adrenals and gonads.^{15,29} Consequently, loss of the cAMP response would be expected to have a negative effect on StAR expression as well as on the expression of other genes in the adrenal cortex.³²

In this study, we focused on the early steps in steroidogenesis. It is certainly possible that knockdown of ACTH-Rs could affect more distal events as well. In fact, in Y1 mouse adrenal cells, ACTH affected the levels of more than 1200 genes, with 46% being upregulated.³² However, nearly all of the inhibition of cortisol observed after siRNA treatment of fetal and adult cells may be accounted for by the marked attenuation of the StAR response because it has been shown in other systems that inhibition of StAR expression is associated with a 75% to 80% reduction in steroid synthesis and secretion.³³

On a percentage basis, the ability of ACTH to stimulate both ACTH-R mRNA and StAR mRNA reduced about twice as much in the adult cells than in the fetal cells. However, even 30% to 40% suppression of the mRNA responses in fetal cells was associated with profoundly reduced cortisol responses to ACTH. It is not clear why the siRNA had a greater effect on the ACTH and StAR responses to stimulation in the adult cells. Nevertheless, the data suggest that a relatively modest reduction in StAR and ACTH mRNA responses is associated with markedly curtailed cortisol secretion.

In control cells transfected with vector containing scrambled RNA, there was almost a 6-fold greater increase in the cAMP responses in the adult cells when compared with the fetal cells. This probably reflects maturation of adenylyl cyclase activity in the adult cells, although differences in ACTH-R number cannot be excluded.^{27,28} The greater magnitude of the ACTH-R mRNA, StAR mRNA responses, and ultimately cortisol secretion seen in the adult cells could all be explained by the enhanced cAMP responses since all have been shown to be stimulated by the cyclic nucleotide.

It is somewhat surprising that although only 25% to 30% of cells were transfected based on the appearance of GFP in them, cAMP and cortisol responses to ACTH were almost completely attenuated. One possible explanation for this is that more cells may have been transfected but at levels too low for detection of the GFP. The evidence argues against a nonspecific effect of transfection being the cause of the loss of cortisol secretion because the control cells were transfected with vector containing scrambled siRNA. These cells had robust responses to stimulation. Also, the responses to stimulation by cells that were not transfected were not different from those observed in cells treated with the control vector (data not shown).

CONCLUSION

We have demonstrated that partial suppression of ACTH-R expression with siRNA markedly attenuates functional responses to stimulation in fetal and adult adrenocortical cells. This observation supports the idea that developmental increases in ACTH-R expression are essential for maturation of cortisol secretory capacity by the adrenal.

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Figure 1.

(A) Phase contrast microscopy of adult adrenal cells in culture after transfection with vector containing small interfering RNA targeted to the adrenocorticotropin (ACTH) receptor and green fluorescent protein (GFP). (B) Fluorescence microscopy of adult adrenal cells in culture after transfection with vector containing small interfering RNA targeted to the ACTH receptor and GFP.

Figure 2.

(Top) Representative blot showing hybridization signals from adrenal corticotrophin receptor (ACTH-R) mRNA protected fragments and 28S rRNA obtained from fetal adrenocortical cells subjected to the treatments indicated. (Bottom) Ratio of ACTH-R mRNA to 28S rRNA in fetal adrenocortical cells subjected to the treatments indicated. Bars identified with different letters are significantly different. In this and subsequent figures, cells from 5 to 9 animals were included in each group ($P < .01$ by Newman-Keuls, all a vs b comparisons). siRNA indicates small interfering RNA.

Figure 3.

(Top) Representative blot showing hybridization signal from steroid acute regulatory protein (StAR) mRNA-protected fragments and 28S rRNA obtained from fetal adrenocortical cells treated as indicated. (Bottom) Ratio of StAR mRNA to 28S rRNA in fetal adrenocortical cells treated as indicated. Bars identified with different letters are significantly different ($P < .05$, a vs b; $P < .01$, a vs c; and $P < .01$, b vs c by Newman-Keuls). siRNA indicates small interfering RNA.

Figure 4.

(A) Cyclic adenosine monophosphate (cAMP) concentration in incubation media that contained fetal adrenocortical cells treated as indicated. Bars identified with different letters are different ($P < .001$, a vs b; $P < .001$, a vs c; $P < .01$, b vs c by Newman-Keuls). (B) Cortisol concentrations in incubation media that contained fetal adrenocortical cells treated as indicated. Bars identified with different letters are different ($P < .01$, a vs b by Newman-Keuls). siRNA indicates small interfering RNA; ACTH, adrenocorticotropin.

Figure 5.

(A, top) Representative blot showing hybridization signals from adrenocorticotropin receptor (ACTH-R) mRNA-protected fragments and 28S rRNA obtained adult adrenocortical cells treated as indicated. (A, bottom) Ratio of ACTH-R mRNA to 28S rRNA in adult adrenocortical cells treated as indicated. Bars identified with different letters are significantly different ($P < .001$, a vs b by Newman-Keuls). (B, top) Representative Western blot of ACTH-R protein obtained from adult adrenal cells. (B, bottom) Optical density signals from Western blots of ACTH-R protein from adult adrenal cells treated as indicated. Bars identified with different subscripts are different ($P < .01$, a vs b; $P = .01$, a vs c; $P < .001$, b vs c by Newman-Keuls). siRNA indicates small interfering RNA.

Figure 6.

(A, top) Representative blot showing hybridization signals from steroid acute regulatory protein (StAR) mRNA-protected fragments and 28S rRNA obtained from adult adrenocortical cells treated as indicated. (A, bottom) Ratio of StAR mRNA to 28S rRNA in adult adrenocortical cells treated as indicated. Bars identified with different letters are different ($P < .001$, a vs b by Newman-Keuls). (B, top) Representative Western blot of StAR protein obtained from adult adrenal cells. (B, bottom) Optical density signals from Western blots of StAR protein from adult adrenal cells treated as indicated. Bars identified with different letters are different ($P < .001$, a vs b; $P < .001$, a vs c; $P < .001$, b vs c by Newman-Keuls). siRNA indicates small interfering RNA; ACTH, adrenocorticotropin.

Figure 7.

(A) Cyclic adenosine monophosphate (cAMP) concentration in incubation media that contained adult adrenocortical cells treated as indicated. Bars identified with different letters are different ($P < .001$, a vs b by Newman-Keuls). (B) Cortisol concentrations in incubation media that contained adult adrenocortical cells treated as indicated. Bars identified with different letters are significantly different ($P < .001$, a vs b by Newman-Keuls). siRNA indicates small interfering RNA; ACTH, adrenocorticotropin.

Figure 8.

(A) Suppression of adrenocorticotropin (ACTH)-induced increase in ACTH receptor and steroid acute regulatory protein (StAR) mRNA in fetal versus adult cells ($P < .01$ by t test).

(B) Suppression of ACTH-induced increased in cyclic adenosine monophosphate and cortisol in fetal versus adult cells ($P < .01$ by t test).

Table 1

Effect of Transfection on Cell Viability

Treatment	Cell Viability
Small interfering RNA	81.68 $\pm$ 0.88
Vector	83.67 $\pm$ 0.88