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ARTICLES

Mechanisms of Inhibition of Dehydroepiandrosterone Upon Corticosterone Release From Rat Zona Fasciculata-Reticularis Cells

Ling-Ling Chang,[1]* Wan-Song Alfred Wun,[2] and Paulus S. Wang[3,4]

[1]Department of Chemical Engineering, Chinese Culture University, Taipei, Taiwan 11114, Republic of China
[2]Obstetrical and Gynecological Associates, P. A., Houston, Texas
[3]Department of Physiology, School of Medicine, National Yang-Ming University, Taipei 11221, Taiwan, Republic of China
[4]Department of Medical Research and Education, Taipei City Hospital, Taipei 10431, Taiwan, Republic of China

Abstract We have demonstrated that dehydroepiandrosterone (DHEA) acts directly on rat zona fasciculata-reticularis (ZFR) cells to diminish corticosterone secretion by an inhibition of post-cAMP pathway, and decreases functions of steroidogenic enzymes after $P450_{scc}$ as well as steroidogenic acute regulatory (StAR) protein expression. However, the mechanisms by which DHEA engages with environmental messenger signals which translate into interfering StAR protein expression are still unclear. This study explored the effects of DHEA on the phosphorylation/activation of extracellular signal-regulated kinases (ERKs). ERK activation resulted in enhancing phosphorylation of steroidogenic factor-1 (SF-1) and increased StAR protein expression. ZFR cells were incubated in the presence or absence of adrenocorticotropin (ACTH), forskolin (FSK), 25-OH-cholesterol, U0126, and H89 at 37°C. The concentration of corticosterone released into the media was measured by radioimmunoassay (RIA). The cells were used to extract protein for Western blot analysis of ERKs or StAR protein expression or immunoprecipitation of SF-1 analysis. The results suggested that (1) ERK pathway of rat ZFR cells might be PKA dependent, (2) ERK activity was required for SF-1 phosphorylation to upregulate steroidogenesis in rat ZFR cells, and (3) DHEA did not affect ERK phosphorylation, however, it attenuated forskolin-stimulated SF-1 phosphorylation to affect StAR protein expression. J. Cell. Biochem. 104: 359–368, 2008.

Key words: ERK; SF-1; DHEA; StAR; zona fasiculata-reticularis cells

Hyperglycemia, hyperinsulinemia, hyperlipidemia, accumulation of excess body fat, and cancer have been attributed to the natural decline in plasma dehydroepiandrosterone-(sulfate) (DHEA(S)) and maintenance of plasma glucocorticoid levels that is associated with aging [Coleman, 1989; Schwartz et al., 1989; Cleary, 1990; Gordon et al., 1991; Berdanier et al., 1993; Svee and Porter, 1998]. Metabolically, DHEA antagonizes the actions of insulin and glucocorticoids by partitioning energy away from fat synthesis and toward oxidation [Berdanier et al., 1993]. These thermogenic properties of DHEA may contribute to its therapeutic potential for the treatment of cardiovascular disease, cancer, obesity, lipemia, and diabetes. Furthermore, DHEA possesses a multi-targeted antioxidant effect [Aragno et al., 1993; Boccuzzi et al., 1997; Brignardello et al., 1998; Bastianetto et al., 1999] and prevents tissue damage induced by acute [Aragno et al., 1997] and chronic hyperglycemia [Aragno et al., 1999; Wellman et al., 1999]. We have reported that DHEA inhibited corticosterone release from rat

L-L. Chang and P.S. Wang contributed equally to this work.

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*Correspondence to: Ling-Ling Chang, PhD, Department of Chemical Engineering, Chinese Culture University, Shih-Lin, Taipei 11114, Taiwan, Republic of China.
E-mail: llchiang@staff.pccu.edu.tw

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adrenal zona fasciculata-reticularis cells (ZFR) in a manner partly related to the post-cAMP pathway by diminishing the steroidogenic enzymes that function subsequent to cytochrome P450 side-chain cleavage enzyme ($P450_{scc}$), and by diminishing steroidogenic acute regulatory (StAR) protein expression [Chang et al., 2003]. However, the mechanisms by which DHEA mediates external signals that interfere with steroidogenesis in rat ZFR cells remain unclear.

The rate-limiting step in steroid biosynthesis is the conversion of cholesterol to pregnenolone by $P450_{scc}$ (see schematic diagram, Fig. 1). In addition to this important enzyme, another protein, StAR, has been identified as the acute regulation mediator of steroid production in steroidogenic tissues. Based on studies for expression of StAR and the observed accompanying increase in steroid biosynthesis [Clark et al., 1994; Stocco and Clark, 1996], the StAR protein represents a most attractive candidate for the transfer of cholesterol from the cytoplasmic pool to the inner mitochondrial membrane. StAR protein expression is regulated by the steroidogenic factor-1 (SF-1/Ad4BP), a transcription factor. The phosphorylated SF-1 (pSF-1) binds to the promoter of the StAR gene and reveals the SF-1 activity through the expression of StAR mRNA [Morohashi and Omura, 1996; Gyles et al., 2001]. On the other hand, SF-1 activity is blocked by DAX-1 (dosage-sensitive sex reversal adrenal hypoplasia congenital, critical region on the X chromosome gene-1), another transcription factor whose expression is mostly restricted to steroidogenic tissues, such as adrenal cortex, ovary, and Leydig cells [Ikeda et al., 1996; Tamai et al., 1996]. The ratio between these two transcription factors may determine whether their combined effects will either enhance or inhibit steroidogenesis in different steroidogenic cell types and at different stages of development. Thus, we questioned whether DHEA inhibited StAR protein expression via diminishing phosphorylation of SF-1.

Recent studies have suggested that the mitogen-activated protein kinase (MAPK) cascade is involved with cellular regulation in steroidogenic activity [Lotfi et al., 2000]. The MAPK family is a focal point of diverse signaling pathways in which the extracellular signal-regulated kinases (ERK1/2), the C-jun N-terminal kinases (JNKs), and the p38 kinases are the upstream kinases in three distinct, yet interacting, signal transduction cascades [Seger and Krebs, 1995]. These protein kinases commonly regulate target gene expression by the

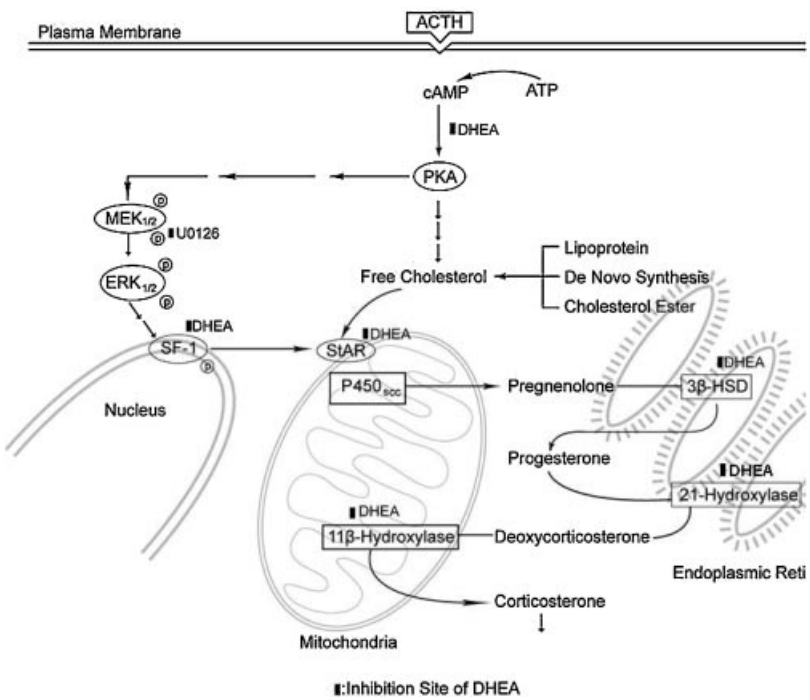

Fig. 1. Schematic representation of the signaling pathways controlling steroidogenesis in rat ZFR cells.

activation of downstream transcription factors. The ERKs include two kinases (p42ERK2 and p44ERK1). ERK1/2 have been implicated in the regulation of SF-1 and activator protein-1 (AP-1) activity in human breast cancer MCF-7 cells and in human kidney COS cells [Hammer et al., 1999]. Upon extracellular stimulation, the ERKs are activated by a network of interacting proteins, which funnel the signal into a multi-tier kinase cascade [Seger and Krebs, 1995; Lewis et al., 1998]. ERK1/2 is activated by a phosphorylation pathway that is regulated by a dual specific tyrosine/threonine kinase, MAPK/ERK kinase (MEK). Therefore, ERK1/2 activation can be selectively blocked by compounds that inhibit MEK activity. By utilizing rat ZFR cells, this study examined (1) whether the ERK pathway regulated the steroidogeneic activity; (2) if DHEA down-regulated the ERK pathway activity; and (3) whether the ERK pathway regulated SF-1 activity. We found that DHEA did not affect ERK phosphorylation, it attenuated forskolin-stimulated SF-1 phosphorylation, and reduced activated StAR protein expression in rat ZFR cells.

MATERIALS AND METHODS

Animals

Female Sprague–Dawley rats weighing 300–350 g (2 months old) provided by National Yang-Ming University were housed in a temperature-controlled room ($22 \pm 1^{\circ}C$) with photoperiods of 14 h (light): 10 h (dark). The light began at 6:00 a.m. Food and water were provided ad libitum. The animals were ovariectomized 4 days before the experiments.

Animal protocols were approved by the Institutional Animal Care and Use Committee of the National Yang-Ming University. All animals received human care in compliance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals, published by the National Science Council, Taiwan, R.O.C.

Reagents

DHEA, bovine serum albumin (BSA), adrenocorticotropin (ACTH), forskolin (FSK), 25-OH-cholesterol, H89, Na-lauroylsarcosine, phenyl-methylsulfonyl fluoride (PMSF), NP-40, Na-deoxycholate, aprotinin, leupeptin, and pepstatin were purchased from Sigma Chemical Co. (St. Louis, MO). The DHEA and 25-OH-cholesterol were dissolved in ethanol as stock solutions. When used to challenge cells, the stock solutions were diluted in Krebs–Ringer bicarbonate buffer with 3.6 mM K^+, 11.1 mM glucose, 0.2% BSA medium (KRBGA). Control studies verified that the dilute ethanol solution was not cytotoxic (data not shown). Proteinase inhibitor cocktail tablets were purchased from Boehringer Mannheim (Mannheim, Germany). Lauryl sulfate (SDS), bromophenol blue, and dithiothreitol were purchased from Research Organics, Inc. (Cleveland, OH). U0126 (1,4-diamino-2,3-dicyano-1,4-bis [2-aminophenylthio]butadiene, a potent and selective non-competitive inhibitor of MAP kinase [Favata et al., 1998]), was provided by Tocris Cookson, Inc. (Ellisville, MO). [^{3}H]-corticosterone was obtained from Amersham Life Science Limited (Buckinghamshire, UK). Anti-StAR antibody was provided by Dr. D. M. Stocco (Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX). Phospho-p44/42 MAP kinase (phosphor-ERK1/2) antibody and p44/p42 MAP kinase (ERK1/2) antibody were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-SF-1 antibody was purchased from Affinity Bioreagents (Golden, CO). Anti-phosphoserine antibody was purchased from Chemicon International, Inc. (Temecula, CA). Protein A/G plus-agarose was purchased from Santa Cruz Biotechnology, Inc. (Santa Crutz, CA). The peroxidase-conjugated IgG fraction to mouse IgG and peroxidase-conjugated IgG fraction to rabbit IgG were purchased from ICN Pharmaceuticals, Inc. (Aurora, OH).

Preparation of Zona Fasciculata-Reticularis (ZFR) Cells for Cell Culture

Rat adrenal glands were excised, and then kept in an ice-cold 0.9% (w/v) NaCl solution. The adipose tissues were removed, and the encapsulated glands were separated by forceps into capsule (mainly zona glomerulosa) and inner zone (mainly ZFR) fractions. The inner zone fractions from 10 to 20 adrenals were pooled for one dispersion, and the ZFR cells were prepared by the procedure of Purdy et al. [1991] with minor modifications [Lo et al., 1998]. The cells (5×10^4 cells/ml) were preincubated with KRBGA medium for 1 h at $37^{\circ}C$ in a shaker bath (50 cycles per min) aerated with 95% O_2 and 5% CO_2. The supernatant was decanted after centrifugation of the tubes at 200*g* for 10 min.

Finally, the cells were resuspended in fresh incubation medium for 20 min, 30 min, 1 h, 3 h, or 5 h. After incubation and centrifugation, the medium was stored at −20°C for corticosterone radioimmunoassay (RIA), and the cells were used for Western blot, RT-PCR, or immunoprecipitation.

In studies examining the effects of kinase inhibitors, the ZFR cells were primed with H89 (an inhibitor of PKA) or U0126 (an inhibitor of MEK) for 30 min, and then further incubated with ACTH, FSK, 25-OH-cholesterol, and DHEA.

Western Blot Analysis

After incubation with or without the appropriate stimulant, ZFR cells were washed twice with ice-cold saline and then harvested in 50 μl of cell lysis buffer (1.5% Na-lauroylsarcosine, 2.5 mM Tris-base, 1 mM EDTA, 0.68% phenylmethylsulfonyl fluoride (PMSF), containing 2% proteinase inhibitors, pH 7.8). Cell lysates were centrifuged for 10 min at 13,000*g*. The supernatant was assayed for protein content [Bradford, 1976] and also was subjected to Western blot analysis [Kau et al., 1999; Lo et al., 2000] to detect StAR, phosphor-ERK1/2, ERK1/2, or β-actin. Samples containing equal amounts of protein were separated by 10% (to detect phosphor-ERK1/2 and ERK1/2) or 12% (to detect StAR and β-actin) acrylamide SDS–PAGE. The relevant proteins were detected on blots using their specific antibodies and were visualized by chemiluminescence (ECL Western blotting detection reagents, Amersham Pharmacia Biotech., Buckinghamshire, UK). The chemiluminescence signal data on X-ray film was evaluated by scanning chemiluminescence pseudo-autogradiograms (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA). Quantification of the scanned images was performed using the ImageQuaNT program (Molecular Dynamics) according to the manufacturer's instructions. The StAR and phosphor-ERK1/2 protein signals were corrected by the β-actin signal and ERK1/2, respectively.

Immunoprecipitation of SF-1

After incubation, the ZFR cells were washed twice with ice-cold saline, then homogenized, and the protein was extracted in 300 μl RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate, 1 mM PMSF, 5 μg/ml aprotinin, 1 mM Na_3VO_4, 1 mM NaF, 5 μg/ml leupeptin, 5 μg/ml pepstatin, and a proteinase inhibitor) on ice. These cell extracts were centrifuged for 10 min at 13,000*g*, and then the protein from the supernatant was incubated with polyclonal SF-1 anti-serum overnight at 4°C. For capture and isolation of immune complexes, the immune complex was incubated with immobilized Protein A/G plus–agarose for 2 h at room temperature. The immunoprecipitated protein was collected by centrifugation at 6,500*g* for 6 min. These immunoprecipitated SF-1 pellets were washed four times with RIPA buffer. After the last wash, the supernatant was discarded and the pellet was resuspended in SDS buffer (0.125 M Tris-base, 4% SDS, 0.001% bromophenol blue, 12% sucrose, and 0.15 M dithiothreitol). Immunoreactivity was detected by polyacrylamide gel electrophoresis and immunoblot analysis using a rabbit anti-phosphoserine primary antibody and a goat anti-rabbit secondary antibody or an anti-SF-1 primary antibody and a goat anti-rabbit secondary antibody. Immunoreactivity was quantified using densitometric scanning of the ECL signal on the film. The phosphorylation of SF-1 protein signal was corrected by SF-1 protein.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

ZFR cells (10^6 cells) were primed with H89 (5 μM), U0126 (50 μM) or vehicle for 30 min, and then incubated with ACTH (0.1 μM), FSK (50 μM), or DHEA (100 μM) for 30 min. RT-PCR analysis was then performed using a previously published method [Chiao et al., 2002]. Following the PCR reaction, tracking dye was added of 10–20 μl to the PCR reaction mixture (100 μl) for analysis by 2% agar [Orly et al., 1994]. The gels were shaken in EtBr solution, washed by water, and gel bands were visualized by UV light. The PCR oligonucleotide primer pairs were designed based on known cDNA sequences of various target genes. The expected PCR products would be 246 bp for rat StAR cDNA [Ronen-Fuhrmann et al., 1998]; and 194 bp for rat RPL19 [Orly and Stocco, 1999]. The fluorescence in each PCR band was normalized to the fluorescence of the RPL19 band.

RIA of Corticosterone

The concentrations of corticosterone in media were determined by RIA as previously described

[Lo et al., 1998; Chang et al., 2002]. With this anti-serum (PSW#4-9), a RIA was established for the measurement of plasma corticosterone levels. The sensitivity of corticosterone RIA was 5 pg/tube. The cross-reactivities were 12% with 11-deoxycorticosterone, 1% with 11-dehydrocorticosterone, 0.3% with aldosterone, and <0.2% with 18-hydroxydeoxycorticosterone, progesterone, estradiol, and testosterone. The intra- and interassay coefficients of variation were 3.3% (n = 5) and 9.2% (n = 4), respectively.

Statistical Analysis

Treatment means were tested for homogeneity using an analysis of variance, and difference between specific means was tested for significance using Duncan's multiple range test or Student's *t*-test [Steel and Torrie, 1960]. A difference between two means was considered statistically significant when P was less than 0.05.

RESULTS

Effects of H89 and U0126 on ACTH-, FSK-, or 25-OH-Cholesterol-Induced Corticosterone Release From ZFR Cells

Exposure of ZFR cells to ACTH (0.001 μM), FSK (10 μM), and 25-OH-cholesterol (10 μM) for 1 h elevated corticosterone secretion by 110-, 12-, and 27-fold, respectively. Priming the cells with either H89 (an inhibitor of PKA, 1 μM) or U0126 (an inhibitor of MEK, 1 or 10 μM) for 30 min prior to incubation with the steroidogenic agents permitted us to examine the role of kinase on corticosterone synthesis. U0126 but not H89 inhibited basal corticosterone release from ZFR cells ($P < 0.01$, Fig. 2). Exposure to either H89 or to U0126 at the higher concentration of 10 μM attenuated ACTH- or FSK-stimulated corticosterone release ($P < 0.05$ or 0.01, Fig. 2) but had no effect on the 25-OH-cholesterol-mediated process.

Effects of U0126 on ACTH-, or FSK-Induced StAR Protein Expression From ZFR Cells

After preincubation, the ZFR cells were further incubated with ACTH (0.1 μM), FSK (10 or 50 μM), or U0126 (10 or 50 μM) for 5 h. ACTH or FSK (50 μM) increased StAR protein expression in ZFR cells ($P < 0.05$, Fig. 3). U0126, at the concentration of 50 μM, attenuated FSK-stimulated StAR protein expression ($P < 0.01$, Fig. 3).

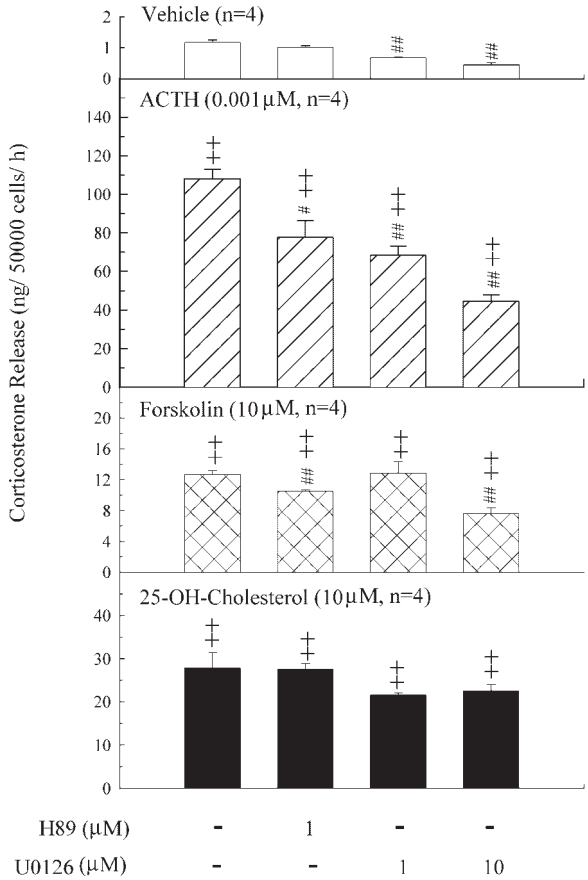

Fig. 2. Effects of H89 or U0126 on the activation of steroidogenesis in ZFR cells by ACTH (0.001 μM), FSK (10 μM), or 25-OH-cholesterol (10 μM). Cultured ZFR cells were primed with H89 (1 μM), U0126 (1 or 10 μM) or vehicle alone for 30 min and thereafter incubated with ACTH (0.001 μM), FSK (10 μM), 25-OH-cholesterol (10 μM), or vehicle alone for an additional 1 h. The corticosterone secreted into the medium was then measured by RIA. $^{++}P < 0.01$ as compared with vehicle group; $^{\#}P < 0.05$ or $^{\#\#}P < 0.01$ as compared with H89 or U0126 = 0 M within the respective group. Each value represents mean ± SEM.

Effects of DHEA on FSK ± H89- or FSK ± U0126-Induced Corticosterone Release From ZFR Cells

The ZFR cells, first primed in the presence of H89 (1 μM), U0126 (10 μM), or vehicle for 30 min, were further incubated with FSK (10 μM) combined with DHEA (0 or 10 μM) for 1 h. DHEA at the concentration of 10 μM diminished the corticosterone release from FSK, FSK + H89, or FSK + U0126 group ($P < 0.01$, Fig. 4). At 10 μM, U0126 significantly reduced the release of corticosterone that occurred in the presence of FSK, or FSK combined with DHEA ($P < 0.01$, Fig. 4). H89 at

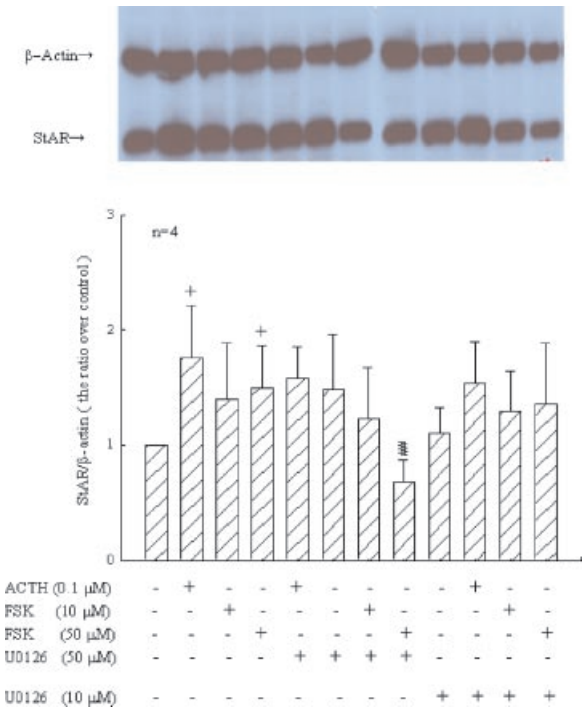

Fig. 3. Effects of U0126 on synthesis of the StAR protein by ZFR cells stimulated with ACTH or FSK. **A**: Western blot analysis of cell extracts subjected to SDS–PAGE and developed by enhanced chemiluminescence. StAR (30 kDa) was detected by incubation with StAR anti-sera. The β-actin served as an internal control. **B**: Quantification of the expression of StAR by the standardization with internal control β-actin. $^{+}P < 0.05$ as compared with vehicle-treated group. $^{\#\#}P < 0.01$ as compared with FSK-treated group. Each value represents mean ± SEM.

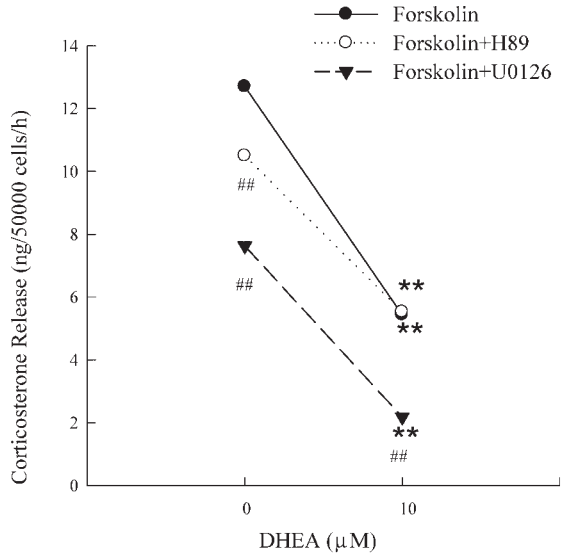

Fig. 4. Effects of DHEA on FSK ± H89- or FSK ± U0126-induced corticosterone release from ZFR cells. Cultured ZFR cells after priming with H89 (1 μM), U0126 (10 μM), or vehicle alone for 30 min were incubated with FSK (10 μM) in combined with DHEA (0 or 10 μM) for 1 h. The corticosterone release into the medium was then measured by RIA. $^{**}P < 0.01$ as compared with DHEA = 0 M within the respective group. $^{\#\#}P < 0.01$ as compared with FSK-treated group. Each value represents mean ± SEM.

this concentration significantly inhibited FSK-mediated corticosterone release ($P < 0.01$, Fig. 4), but had no effect when FSK was combined with DHEA.

Effects of U0126 or H89 on ACTH-, FSK-, or DHEA-Activation of the Phosphorylation of ERK1/2 Expression From ZFR Cells

Treatment of ZFR cells with FSK (50 μM) resulted in transient activation of ERK1/2, and phosphorylation of ERK1/2 was detectably elevated within 5 min and decayed beyond 30 min (data not shown). The ZFR cells, after priming with H89 (5 μM), U0126 (50 μM), or vehicle for 20 min, were further incubated with ACTH (0.1 μM), FSK (50 μM), or DHEA (100 μM) for 20 min. No apparent increase in the phosphorylation of ERK1/2 was induced by ACTH or DHEA as compared with basal levels (data not shown). Although the mean phosphorylation levels of ERK1/2 rose with 50 μM FSK, the differences were not statistically significant. Similarly, H89 had no significant effect on phosphorylation levels of ERK1/2 in FSK-treated cells (data not shown). However, the MEK-specific inhibitor U0126 significantly reduced the level of phosphorylation of ERKs in the presence of FSK or DHEA ($P < 0.05$ or 0.01, respectively, data not shown).

Effects of U0126 or H89 on FSK- or DHEA-Activation of Phosphorylation of SF-1 Expression From ZFR Cells

Preincubated ZFR cells were exposed to with FSK (50 μM), DHEA (100 μM), H89 (5 μM), or U0126 (50 or 150 μM) for 3 h. FSK treatment resulted in a significant increase in the phosphorylation of SF-1 ($P < 0.01$, Fig. 5). DHEA, H89, or U0126 (150 μM) all significantly reduced the FSK-activated phosphorylation of SF-1 ($P < 0.05$ or 0.01, Fig. 5). U0126 at the lower concentration of 50 μM had no significant effect on FSK-activated phosphorylation of SF-1 (Fig. 5).

U0126 at concentrations of 10, 50, and 150 μM significantly attenuated FSK-stimulated corticosterone release, StAR protein expression, and phosphorylation of SF-1 expression (Figs. 2,3 and 5), respectively. Control studies indicated

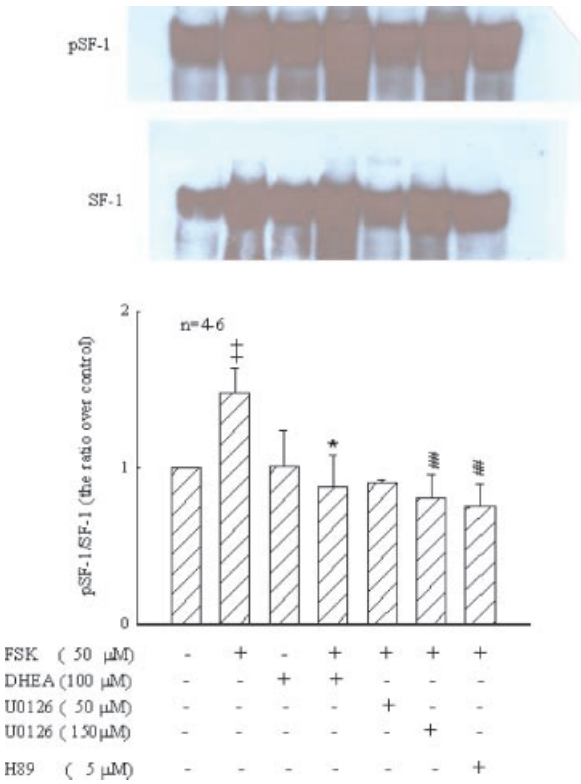

Fig. 5. Effects of U0126 or H89 on FSK-, or DHEA-activated phosphorylation of SF-1 from ZFR cells. ZFR cells were incubated with FSK (50 μM) or DHEA (100 μM) in the presence of H89 (0 or 5 μM), U0126 (0, 50 or 150 μM) for 3 h. The cell pellets after immunoprecipitation with SF-1 antibody were immunoblotted with anti-phosphoserine antibody or anti-SF-1 antibody. Immunoreactivity was quantified using densitometric scanning of the ECL signal on the film. The phosphor-SF-1 protein (pSF-1) signal was corrected by SF-1. $^{++}P < 0.01$ as compared with vehicle-treated group. $^{*}P < 0.05$ as compared with DHEA = 0 M within the respective group. $^{\#\#}P < 0.01$ as compared with H89 or U0126 = 0 M within the respective group. Each value represents mean ± SEM.

that when ZFR cells were incubated with U0126, and then were challenged with ACTH (0.001 μM) alone, the corticosterone secretion increased. There were no differences in the levels of corticosterone secretion between the basal (no U0126) and the U0126 groups (10–150 μM; data not shown). The media collected from ZFR cells incubated in the absence or presence of U0126 (10–150 μM) were assayed for lactate dehydrogenase (LDH). The LDH levels were not significantly different between the basal and the U0126 groups (data not shown), suggesting no adverse effects on the cell membrane. The above observations indicate that the inhibitory effects of U0126 were not due to non-specific toxic phenomena.

Effects of U0126 or H89 on FSK- or DHEA-Activation of StAR mRNA Expression From ZFR Cells

After priming ZFR cells with H89 (5 μM), U0126 (50 μM), or vehicle for 30 min, these cells were further incubated with ACTH (0.1 μM), FSK (50 μM), or DHEA (100 μM) for 30 min. There were no significant differences in StAR mRNA levels among these treatments (data not shown).

DISCUSSION

ACTH is the major hormone which regulates not only acute glucocorticoid secretion, but also the expression and maintenance of adrenal cell specific functions, that is, regulation of its receptor number [Penhoat et al., 1994], the expression of the genes encoding the steroidogenic enzymes [Simpson and Waterman, 1988], and the cAMP second messenger systems which activate cAMP-dependent protein kinase A (PKA) [Schimmer, 1980; Clegg et al., 1992; Wong et al., 1992]. The most highly characterized mechanism of action of FSK is activation of adenylate cyclase, which leads to elevation of intracellular cAMP. The cyclic nucleotide serves as a second messenger for the upregulation of the StAR protein. In this study, either ACTH or FSK increased corticosterone secretion (Fig. 2) as well as StAR protein expression level (Fig. 3). H89 attenuated either ACTH- or FSK-induced corticosterone release (Fig. 2).

The interaction between the cAMP/PKA and the ERK cascades has been studied in several cellular systems [Bornfeldt and Krebs, 1999]. In some study systems, such as in epidermal growth factor-stimulated Rat1 fibroblasts [Wu et al., 1993] or platelet-derived growth factor-stimulated human arterial smooth muscle cells [Graves et al., 1993], cAMP inhibited the activation of the ERK cascade. In other cell systems, such as nerve growth factor-stimulated PC12 cells, cAMP activated ERK to induce various mitogens or differentiation processes [Vossler et al., 1997]. In the present work, H89 did not significantly eliminate the FSK-mediated phosphorylation of ERK. Therefore, we cannot rule out the possibility that the ERK cascade is also modulated by an alternative signaling mechanism such as cAMP responsive guanine nucleotide exchange factors (Epac1 and Epac2). Upon binding cAMP, Epac1/2 rapidly active Rap1, which subsequently promotes activation of

B-Raf and the rest of the ERK cascade [de Rooij et al., 1998].

U0126 significantly decreased the corticosterone release under basal, ACTH-, and FSK-stimulated conditions (Fig. 2) suggesting the involvement of the ERK cascade in steroidogenesis. This MEK-specific inhibitor also attenuated phosphorylation of ERK1/2 in the presence of FSK (data not shown), but had no inhibitory effect on the release of corticosterone from ZFR cells by 25-OH-cholesterol supplementation (Fig. 2). 25-OH-cholesterol passes unassisted from the outer mitochondrial membrane to the inner mitochondrial membrane (IMM), where it is converted to pregnenolone by $P450_{scc}$. These observations suggested that the MEK inhibitor (U0126) did not interfere with $P450_{scc}$ function and the logical site(s) of action for ERK1/2 should be the delivery of cholesterol before the IMM stage (Fig. 1).

Reports examining the involvement of ERK1/2 cascade in the regulation of steroidogenesis are contradictory, with some observing stimulatory effects [Cameron et al., 1996; Das et al., 1996; Gyles et al., 2001] and others reporting inhibitory effects [Tajima et al., 2003]. For example, Gyles et al. [2001] demonstrated that MAPK activation led to an increase in steroidogenesis in Y1 adrenocortical cells. However, Tajima et al. [2003] proposed that gonadotropin activated MAPK and thereby down-regulated steroidogenesis in human and rat preovulatory granulosa cells. By using rat ZFR cells, we observed an increase in the phosphorylation of ERK1/2 in response to FSK, but the change was not significant (data not shown). Moreover, U0126 specifically diminished StAR protein expression from FSK-primed ZFR cells ($P < 0.01$, Fig. 3) but with no elevation of accompanying StAR mRNA expression (data not shown). Thus, these data are suggestive, but not conclusive, that activation of MAPK cascade by FSK might serve as a novel mechanism to upregulate steroidogenesis via activation of StAR protein expression.

SF-1 is a critical transcription factor in the induction of steroidogenesis and is believed to bind and activate the promoter of the StAR gene in its phosphorylated form pSF-1 [Morohashi and Omura, 1996; Gyles et al., 2001]. In this study, FSK increased the phosphorylation of SF-1 as assessed by SF-1 immunoprecipitation and phosphoserine immuoblotting ($P < 0.01$, Fig. 5). However, MEK inhibition by U0126 caused a reduction in FSK-stimulated phosphorylation of SF-1 level ($P < 0.01$, Fig. 5). Taken together, the data suggest that the upregulation of StAR occurs via the activation of the MAPK cascade and the subsequent induction of phosphorylation of SF-1 leads to the de novo synthesis of StAR protein and enhanced steroidogenesis.

Our previous report [Chang et al., 2003] showed DHEA inhibited corticosterone release from ZFR cells by decreasing cAMP production, StAR protein expression, $P450_{scc}$ activity, and 11β-hydroxylase activity. DHEA could further diminish the corticosterone release from cells suppressed by FSK + U0126 ($P < 0.01$, Fig. 4). DHEA inhibited FSK-induced corticosterone production and its inhibition was additive with the effects of U0126 ($P < 0.01$, Fig. 4). This observation suggests the two inhibition mechanisms/pathways were either independent or separated by a long gap on the same action cascade. H89 suppressed FSK-induced corticosterone release ($P < 0.01$, Fig. 4), and the addition of H89 had no further significant effect on the corticosterone release from FSK + DHEA group (Fig. 4). These results suggest that DHEA and H89 act either through similar pathway(s)/mechanism(s) or at very closely located sites on the same action cascade. DHEA alone did not affect ERK1/2 phosphorylation, but U0126 attenuated ERK1/2 phosphorylation in the presence of DHEA. DHEA may be involved in the ERK1/2 pathway, however, DHEA did not inhibit StAR expression by decreasing ERK1/2 phosphorylation. DHEA may attenuate FSK-stimulated phosphorylation of SF-1 expression (Fig. 5) but not StAR mRNA expression (data not shown). Taken together, our data suggest that the DHEA inhibition of StAR protein expression is mediated through the down-regulation of StAR protein expression via the inhibition of PKA activity, which, through the MAPK cascade, interferes with the phosphorylation of SF-1, thus leading to the diminished expression of the StAR protein and decreased steroidogenesis.

In summary, the present results propose a link between the PKA activation through the ERK cascades, thus upregulating steroidogenesis via the induction of phosphorylation of SF-1, leading to the de novo synthesis of the StAR protein and enhanced corticosterone release from rat ZFR cells. DHEA inhibited the PKA activity through the ERK cascades to decrease

the FSK-stimulated phosphorylation of SF-1, thereby inhibiting the corticosterone release.

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