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Effects of Evodiamine and Rutaecarpine on the Secretion of Corticosterone by Zona Fasciculata-Reticularis Cells in Male Rats

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ABSTRACT

Evodiamine (EVO) and rutaecarpine (RUT) are two bioactive alkaloid isolated from Chinese herb named Wu–Chu–Yu. Previous studies have shown that EVO and RUT possess thermoregulation, vascular regulation, anti-allergic, anti-nociceptive and anti-inflammatory activities. The mechanisms of EVO and RUT effect on steroidogenesis are not clear. The goal of this study was to characterize the mechanism by which EVO and RUT affect corticosterone production in rat zona fasciculata-reticularis (ZFR) cells. ZFR cells were isolated from adrenal glands of male rats and incubated with adrenalcorticotropin (ACTH, 10^{-9} M), forskolin (an adenylyl cyclase activator, 10^{-5} M), 8-bromo-adenosine 3′:5′-cyclic monophosphate (8-Br-cAMP, a permeable cAMP analog, 10^{-4} M), or steroidogenic precursors including 25-hydroxycholesterol, pregnenolone, progesterone, and deoxycorticosterone, 10^{-5} M each, in the presence or absence of EVO and RUT respectively (0–10^{-3} M) at 37°C for 1 h. The concentrations of corticosterone, pregnenolone and progesterone in the media were measured by radioimmunoassay. After administration of ZFR cells with EVO or RUT (10^{-4} M) for 60 and 120 min, Western blot analysis was employed to explore the influence of EVO and RUT on the expression of cytochrome P450 side chain cleavage enzyme (P450scc) and steroidogenic acute regulatory protein (StAR). EVO and RUT reduced both basal and ACTH-, forskolin-, as well as 8-Br-cAMP-stimulated corticosterone production in rat ZFR cells. The enhanced corticosterone production caused by the administration of four steroidogenic precursors was decreased following EVO or RUT challenge. These results suggest that EVO and RUT inhibit corticosterone production in rat ZFR cells via a mechanism involving: (1) a decreased activity of cAMP-related pathways; (2) a decreased activity of the steroidogenic enzymes, that is, 3β-hydroxysteroid dehydrogenase (3β-HSD) and 11β-hydroxylase (P450c11), during steroidogenesis; and (3) an inhibition of StAR protein expression. J. Cell. Biochem. 108: 469–475, 2009.

KEY WORDS: EVO; RUT; P450scc; StAR

Wu–Chu–Yu (Fructus evodiae) is a Chinese herb purified from the dried, unripe fruit of *Evodia rutaecarpa* (*E. rutaecarpa*). Two major bioactive alkaloids, EVO and RUT (Fig. 1) were isolated and used for cold hand, migraines, and vomiting etc. EVO has been reported to affect many physiological functions including vasorelaxation, uterotonic action, anoxia, and control of body temperature [Yamahara et al., 1989; Chiou et al., 1992, 1997; Tsai et al., 1995]. EVO decreased testosterone production via a reduction of 17β-hydroxysteroid dehydrogenase activity in testicular interstitial cells [Lin et al., 1999] and aldosterone secretion via an inhibitory effect on 11β-hydroxylase in glomerulosa cells [Hung et al., 2001]. Ethanol extract of *E. rutaecarpa* and its bioactive components exhibited anti-inflammatory activities [Ko et al., 2007]. RUT, a quinazolinocarboline alkaloid, exerts extensive biological and pharmacological activities, such as inhibiting inflammatory responses, lowering blood pressure, dysmenorrheal, gastrointestinal

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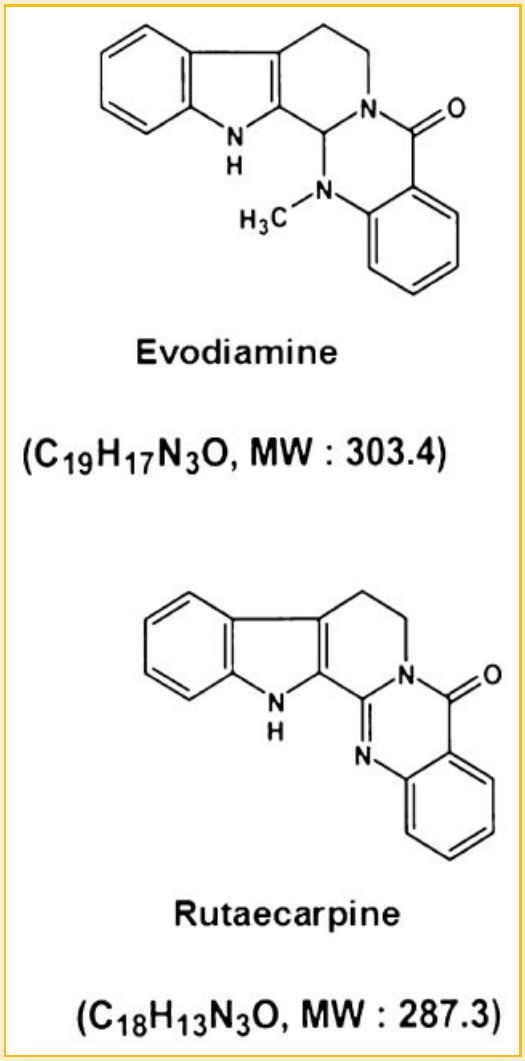

Fig. 1. The chemical structures of evodiamine and rutaecarpine isolated from Wu-Chu-Yu.

disorders and protecting against I/R-induced myocardial damages [Choi et al., 2006]. Pharmacological studies indicate that RUT has various bioactivities, such as causing vasodilatation by mechanisms of an endothelium-dependent manner coupled with the synthesis or release of nitric oxide [Wang et al., 1996], inducing positive inotropic and chronotropic actions [Kobayashi et al., 2001], suppressing platelet plug formation in mesenteric venules [Sheu et al., 2000], gastro-protective effect against injury induced by aspirin and stress [Wang et al., 2005], and inhibition of COX-2 [Moon et al., 1999]. The purpose of this study was to investigate the mechanism by which EVO and RUT affect corticosterone production in rat ZFR cells.

MATERIALS AND METHODS

ANIMALS

Male Sprague–Dawley rats weighing 300–350 g were housed in a temperature-controlled room ($22 \pm 1^{\circ}C$) with 14 h of artificial illumination daily (0600–2000). Food and water were given ad libitum. All animal experimentation has been conducted humanely and in conformance with the policy statement of the Committee of National Yang-Ming University.

PREPARATION OF ZFR CELLS FOR CELL CULTURE

An adrenocortical preparation enriched with zona fasciculata-reticularis (ZFR) cells for culture was performed following a method described by Lo et al. [1998a]. Male Sprague–Dawley rats were decapitated. The adrenal glands were rapidly excised and stored in an ice-cold 0.9% NaCl solution. The adipose tissues were removed. The encapsulating glands were separated into capsule (mainly zona glomerulosa) and inner zone (mainly ZFR) fractions with forceps. The fractions of inner zone from 10 to 20 adrenals were incubated with collagenase (2 mg/ml, Sigma Chemical) at $37^{\circ}C$ in a shaking water bath, 100–110 strokes/min, for 60 min. The collagenase was dissolved in 2–4 ml of Krebs-Ringer bicarbonate buffer (3.6 mM K^{+}/l, 11.1 mM glucose/L) with 0.2% bovine serum albumin (BSA) medium (KRBGA), pH 7.4. ZFR cells were dispersed by repeated pipetting and filtered through a nylon mesh. After centrifugation at 200*g* for 10 min, the cells were washed with KRBGA medium and centrifuged again. Erythrocytes were eliminated from ZFR cells by washing with 4.5 ml distilled water for a few seconds. The ZFR cells were then mixed with 0.5 ml of 10× Hanks' balanced salt solution (pH 7.4). After centrifugation at 200*g* for 10 min, the supernatant was discarded, and the pellet was resuspended in 3 ml of KRBGA solution. An aliquot (20 μl) was used for cell counting in a hemocytometer after staining with 0.05% nigrasin stain. Cells in culture medium were further diluted to a concentration of $5–10 \times 10^{4}$ cells/ml and divided into the test tubes.

PREPARATION OF EVO AND RUT

The ethanol extract of *E. rutaecarpa* was prepared as described elsewhere [Ko et al., 2002, 2003]. After being vacuum-dried, the ethanol extract of *E. rutaecarpa* was re-dissolved in dimethyl sulfoxide (DMSO) as a 10 mg/ml stock solution. The two bioactive components, evodiamine and rutaecarpine were purified from *E. rutaecarpa*. Their identities were confirmed by comparing their NMR and IR spectra with those reported in the literature [Lin et al., 1991].

IN VITRO EXPERIMENTS

The ZFR cells were incubated in KRBGA medium with 1 ml/tube for 60 min at $37^{\circ}C$ under 95% O_2–5% CO_2. To measure the effects of EVO and RUT on the secretion of corticosterone by male rat ZFR cells when reacted with ACTH, forskolin, 8-Br-cAMP, steroidogenic precursors include 25-hydroxy-cholesterol (25-OH-C), pregnenolone, progesterone and deoxycorticosterone, ZFR cells were preincubated with one or more substances respectively for 60 min in KRBGA medium. After incubation and centrifugation at 200*g* for 10 min, the supernatant was used to measure the concentrations of corticosterone, pregnenolone and progesterone by RIA.

RIA OF CORTICOSTERONE

The concentrations of corticosterone in plasma extract and media were determined by RIA as described elsewhere [Chen et al., 1997; Lo et al., 1998a], with anticorticosterone serum (PSW#4-9, by

Dr. P.S. Wang, NYMU, Taipei, Taiwan); the sensitivity of corticosterone detection was 5 mg per assay tube. The intra- and interassay coefficients of variation were 3.3% (n = 5) and 9.2% (n = 4), respectively.

RIA OF PREGNENOLONE

The concentration of pregnenolone in media was determined by RIA as previously described [Kau et al., 1999; Chang et al., 2002]. The anti-pregnenolone antiserum was purchased from Biogenesis, Inc. (Sandown, NH). The sensitivity of pregnenolone RIA was 16 pg per tube. The concentration of pregnenolone was determined by RIA with anti-pregnenolone antiserum purchased from Biogenesis, Inc. The sensitivity of the pregnenolone RIA was 16 pg per assay tube. The intra- and interassay coefficients of variation were 2.3% (n = 6) and 3.7% (n = 4), respectively.

RIA OF PROGESTERONE

The concentration of progesterone in plasma samples was determined by RIA as described elsewhere using antiprogesterone serum W-5 [Lu et al., 1996]. With anti-progesterone W-5, the sensitivity of progesterone RIA was 5 pg per assay tube. The cross-reactivities were 8% with testosterone, androstenedione, and pregnenolone, 2.5% with 5α-dihydrotestosterone, 2% with 17 alpha-hydroxyprogesterone, and ~0.3% with estrone, 17α-estradiol, 17β-estradiol, estriol, cortisone, hydrocortisone, and cholesterol. The intra- and interassay coefficients of variability (CV) were 4.8 (n = 5) and 9.5% (n = 4), respectively [Lu et al., 1998].

GEL ELECTROPHORESIS AND WESTERN BLOTTING

The Western blotting method has been reported previously [Lo et al., 1998b; Kau et al., 1999]. ZFR cells were washed twice with ice-cold 0.9% NaCl, followed by extraction for 30–60 s on ice with homogenization buffer, pH 8.0, containing 1.5% Na-Lauroylsacrosine, 2.5 mM Tris-base, 1 mM EDTA, 0.68% phenylmethylsulfonyl fluoride (PMSF), and 2% proteinase inhibitors using an ultrasonic sonicator (model XL 2020, Heat Systems, Inc., Farmingdale, NY). Cell mixtures were centrifuged for 12 min at 13,000 rpm. The protein concentration in the supernatant was determined by the Bradford method [Bradford, 1976]. Extracted proteins were denatured by boiling for 10 min in SDS buffer (0.125 M Tris-base, 4% sodium dodecyl sulfate (SDS), 0.001% bromophenol blue, 12% sucrose, and 0.15 M dithiothreitol) [Hu et al., 1991]. The proteins in the samples were separated using 12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) at 50 V for 20 min and then at 100 V for 60 min using a running buffer. The proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (NEN Life Science Products, Boston, MA) using a Trans-Blot SD semi-dry-transfer cell (170-3940, Bio-Rad, Hercules, CA) at 60 mA (for 8- to 10-mm membrane) for 40 min in a transfer solution. The membranes were washed in buffer (TBS-T buffer, containing 0.8% NaCl, 0.02 M Trisbase, and 0.3% Tween-20, pH 7.6) for 5 min and then blocked by a 120-min incubation in blocking buffer (TBS-T buffer containing 5% nonfat dry milk). These membranes were incubated with anti-StAR protein antibody (1:2,000, rabbit) or anti-P450scc antibody (1:2,000, rabbit) in 5% nonfat dry milk of TBS-T buffer overnight at 4°C. After three washes with TBS-T buffer that were 15, 5, and 5 min respectively, the membranes were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG, 1:8,000 dilution) in 5% nonfat dry milk of TBS-T buffer. The membranes were washed three times with TBS-T buffer, then the band for StAR protein or P450scc was visualized by chemiluminescence (ECL reagent Kit, Amersham, UK). The P450scc and StAR protein signals were corrected by β-actin signal.

STATISTICAL ANALYSIS

The treatment means of both in vivo and in vitro studies were tested for homogeneity using analysis of variance (ANOVA), and the difference between specific means was tested for significance using Duncan's multiple range test. A difference between two means was considered statistically significant when $P < 0.05$.

RESULTS

EFFECTS OF RUT AND EVO ON THE BASAL, ACTH, FORSKOLIN, AND 8-Br-cAMP STIMULATED CORTICOSTERONE SECRETION BY MALE RAT ZFR CELLS

Basal corticosterone secretion by male rat ZFR cells was stimulated by ACTH (10^{-9} M) ($P < 0.01$), however this was inhibited by addition of rutaecarpine (10^{-6} to 10^{-3} M) and evodiamine respectively (10^{-6} to 10^{-3} M) ($P < 0.01$). Forskolin (10^{-5} M) and 8-Br-cAMP (10^{-4} M) stimulated corticosterone secretion by male rat ZFR cells ($P < 0.01$). The reaction was inhibited by addition of RUT (10^{-6} to 10^{-3} M) and EVO (10^{-6} to 10^{-3} M) ($P < 0.01$) (Fig. 2).

EFFECTS OF EVO ON CORTICOSTERONE SECRETION IN RESPONSE TO STEROIDOGENIC PRECURSORS BY RAT ZFR CELLS

Corticosterone secretion by rat ZFR cells increased in response to steroidogenic precursors with different concentrations (10^{-7} M, 10^{-5} M, $P < 0.01$). The precursors include 25-hydroxycholesterol (25-OH-C), pregnenolone (Δ_5P), progesterone (P_4) and deoxycorticosterone (DOC). However, this reaction was inhibited by addition of RUT (10^{-4} M) and EVO (10^{-4} M) respectively, ($P < 0.01$) (Figs. 3 and 4).

EFFECT OF EVO AND RUT ON THE ACTIVITY OF P450scc IN ZFR CELLS OF MALE RATS

The release of pregnenolone by ZFR cells of male rats increased with different concentrations of 25-OH-cholesterol (10^{-7} M, 10^{-5} M, $P < 0.01$). This reaction was not inhibited by addition of RUT (10^{-4} M) and EVO (10^{-4} M) respectively (Fig. 5).

EFFECT OF EVO AND RUT ON THE ACTIVITY OF 3β-HYDROXYSTEROID DEHYDROGENASE (3β-HSD) IN RAT ZFR CELLS

The release of progesterone by ZFR cells of male rats increased with different concentrations of pregnenolone (10^{-7} M, 10^{-5} M, $P < 0.01$). However, this reaction was inhibited by addition of RUT (10^{-4} M) and EVO (10^{-4} M) respectively, ($P < 0.01$) (Fig. 6).

EFFECTS OF EVO AND RUT ON StAR AND P450scc PROTEIN EXPRESSIONS IN MALE RAT ZFR CELLS

EVO (10^{-4} M) did not influence the P450scc protein expressions in male rat ZFR cell. EVO also did not influence the StAR protein

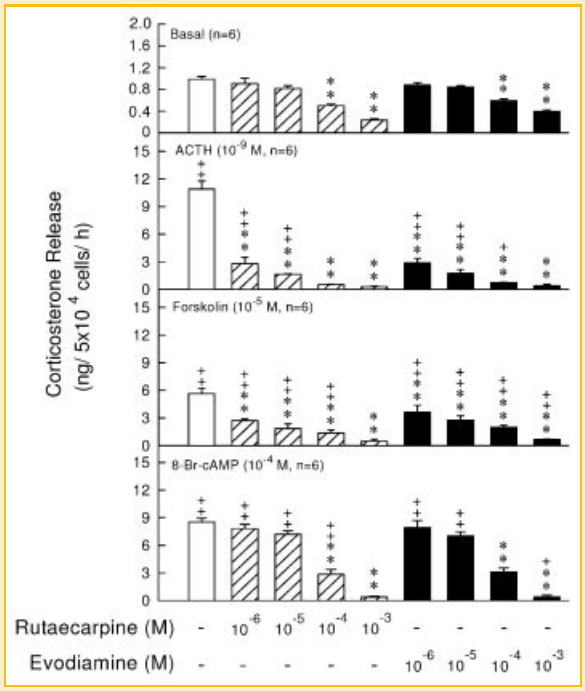

Fig. 2. Effects of rutaecarpine (10^{-6} to 10^{-3} M) and evodiamine (10^{-6} to 10^{-3} M) on the basal, ACTH (10^{-9} M)-, forskolin (10^{-5} M)-, and 8-Br-cAMP (10^{-4} M)-stimulated corticosterone secretion by male rat ZFR cells. $^{*}P<0.05$, $^{**}P<0.01$ compared with rutaecarpine or evodiamine = 0 M, respectively. $^{+}P<0.05$, $^{++}P<0.01$ as compared with basal. Each value represents mean ± SEM.

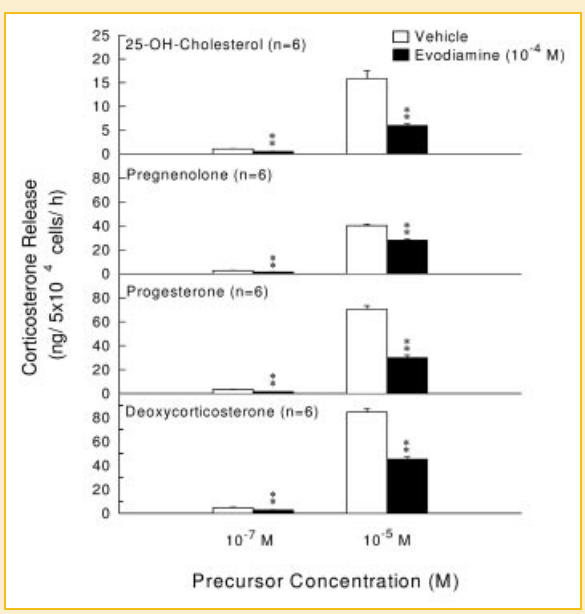

Fig. 3. Effects of evodiamine on corticosterone secretion in response to steroidogenic precursors by rat ZFR cells. The precusors include 25-hydroxy-cholesterol (25-OH-C), pregnenolone (Δ_5P), progesterone (P_4) and deoxycorticosterone (DOC). $^{**}P<0.01$ as compared with vehicle group. Each value represents mean ± SEM.

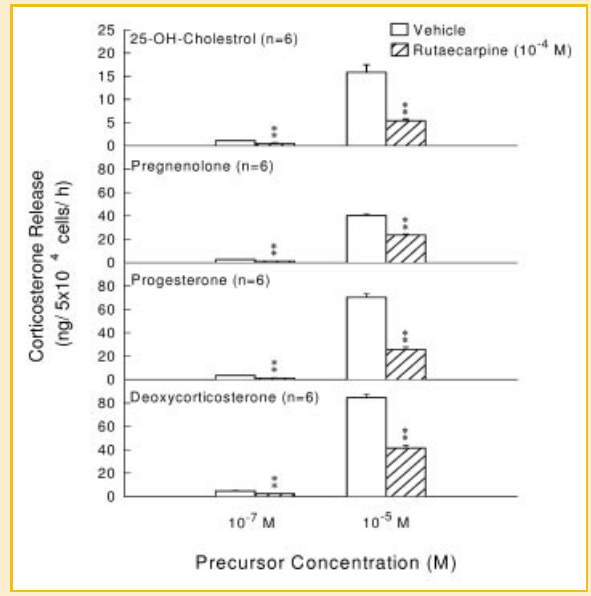

Fig. 4. Effects of rutaecarpine on corticosterone secretion in response to steroidogenic precursors by rat ZFR cells. The precusors include 25-hydroxy-cholesterol (25-OH-C), pregnenolone (Δ_5P), progesterone (P_4), and deoxy-corticosterone (DOC). $^{**}P<0.01$ as compared with vehicle group. Each value represents mean ± SEM.

expressions in male rat ZFR cell by 60 min incubation. However, EVO inhibited the StAR protein expression after 120 min incubation ($P<0.05$) (Figs. 7–9). RUT (10^{-4} M) did not influence the P450scc protein expressions in male rat ZFR cell. RUT also did not influence the StAR protein expressions in male rat ZFR cell by 60 min incubation. However, RUT inhibited the StAR protein expression after 120 min incubation ($P<0.05$) (Figs. 10–12).

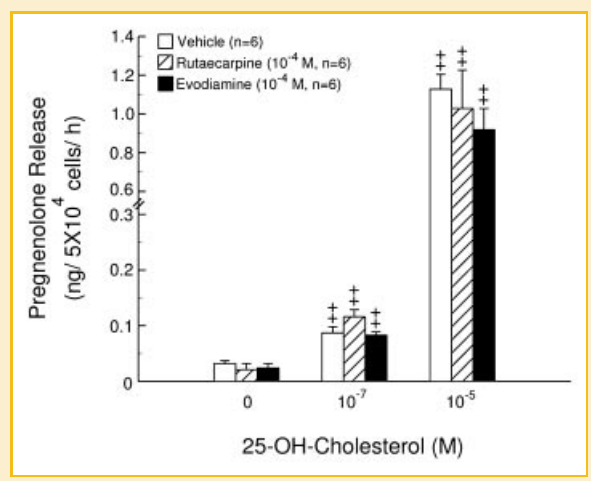

Fig. 5. Effect of evodiamine (10^{-4} M) and rutaecarpine (10^{-4} M) on the activity of P450scc in ZFR cells of male rats. $^{++}P<0.01$ as compared with evodiamine or rutaecarpine = 0 M. Each value represents mean ± SEM.

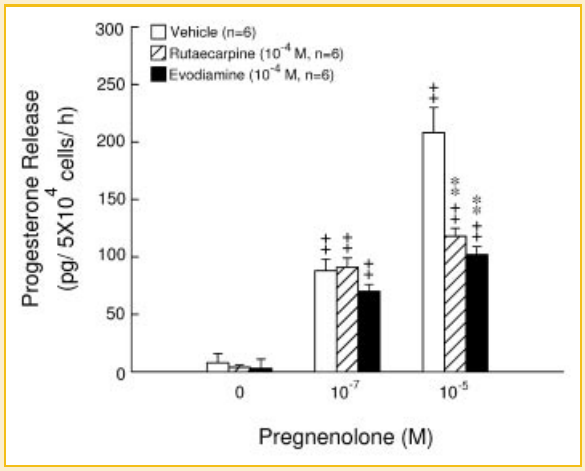

Fig. 6. Effect of evodiamine (10^{-4} M) and rutaecarpine (10^{-4} M) on the activity of 3β-HSD in ZFR cells of male rats. $^{**}P < 0.01$ as compared with vehicle group. $^{++}P < 0.01$ as compared with evodiamine or rutaecarpine = 0 M. Each value represents mean ± SEM.

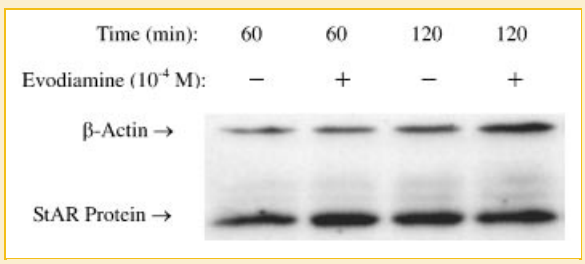

Fig. 7. Effects of evodiamine on StAR protein expression in male rat ZFR cells. The ZFR cells pretreated with vehicle or evodiamine for 60 or 120 min. The expressions of StAR protein were analyzed by Western blot.

DISCUSSION

Basal corticosterone secretion by male rat ZFR cells was stimulated by ACTH (10^{-9} M) ($P < 0.01$) (Fig. 2), however this was strongly inhibited by addition of RUT and EVO, respectively (10^{-6} to 10^{-3} M, $P < 0.01$). It has been shown that EVO decreased the basal level of and angiotension II-induced release of aldosterone in rat zona glomerulosa cells via reducing the activity of 11β-hydroxylase during the steroidogenesis of aldosterone [Hung et al., 2001].

Forskolin (an adenylyl cyclase activator) and 8-Br-cAMP (analog of cAMP) stimulated corticosterone secretion by male rat ZFR cells.

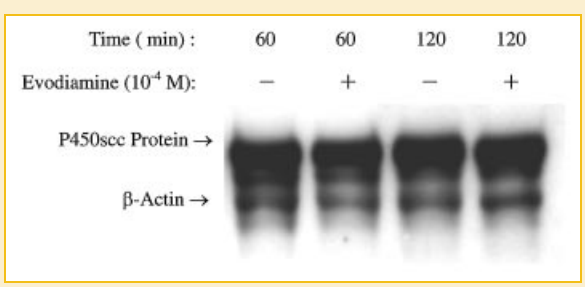

Fig. 8. Effects of evodiamine on P450scc protein expression in male rat ZFR cells. The ZFR cells pretreated with vehicle or evodiamine for 60 or 120 min. The expressions of P450scc protein were analyzed by Western blot.

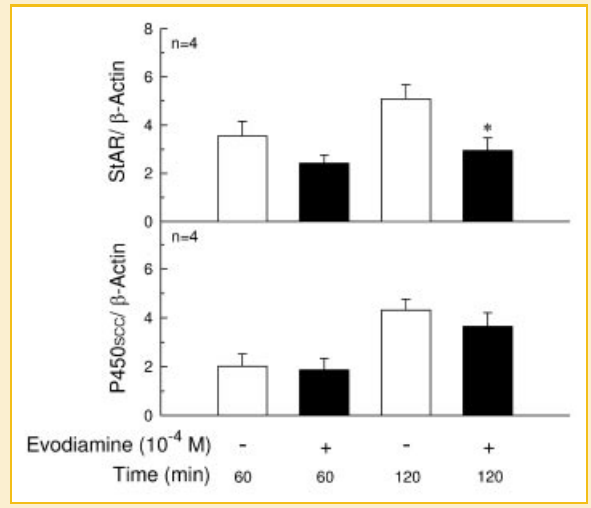

Fig. 9. Effects of evodiamine on StAR protein and P450scc protein expression in male rat ZFR cells. $^{*}P < 0.05$ as compared with vehicle group. Each value represents mean ± SEM.

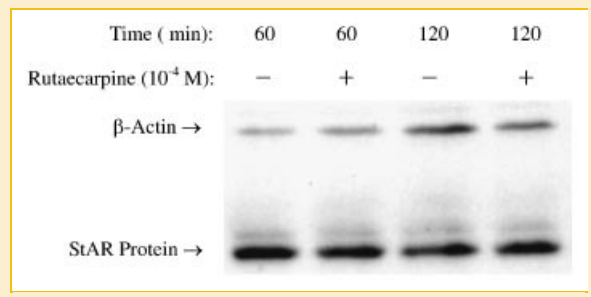

Fig. 10. Effects of rutaecarpine on StAR protein expression in male rat ZFR cells. The ZFR cells pretreated with vehicle or rutaecarpine for 60 or 120 min. The expressions of StAR protein were analyzed by Western blot.

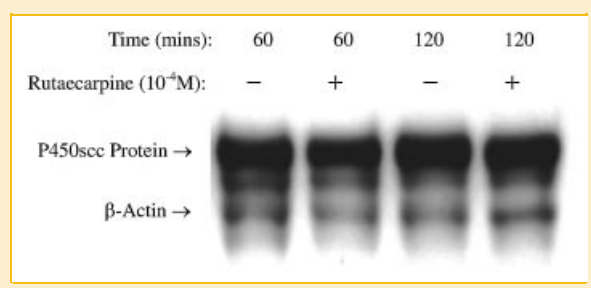

Fig. 11. Effects of rutaecarpine on P450scc protein expression in male rat ZFR cells. The ZFR cells pretreated with vehicle or rutaecarpine for 60 or 120 min. The expressions of P450scc protein were analyzed by Western blot.

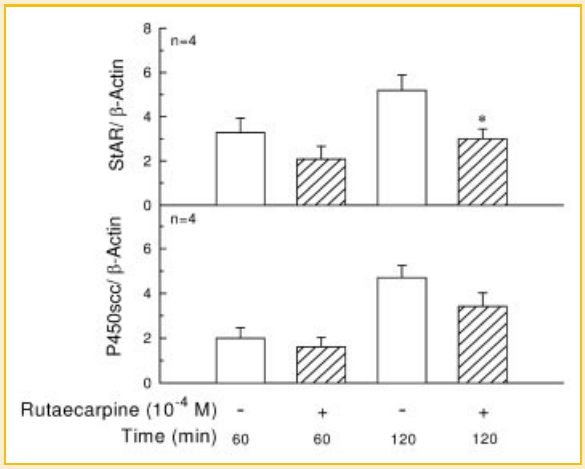

Fig. 12. Effects of rutaecarpine on StAR protein and cytochrome P450scc protein expression in male rat ZFR cells. $^{*}P < 0.05$ as compared with vehicle group. Each value represents mean ± SEM.

However, this reaction was inhibited by addition of RUT (10^{-6} to 10^{-3} M) or EVO (10^{-6} to 10^{-3} M) (Fig. 2). These indicated both Rut and EVO inhibited corticosterone secretion by male rat ZFR cells via cAMP pathway. It has been well established that EVO decreased forskolin-, 8-Br-cAMP- and androstenedione-stimulated testosterone secretion in testicular interstitial cells via a mechanism involving reduced activity of cAMP-related pathways and 17β-hydroxysteroid dehydrogenase (17β-HSD) [Lin et al., 1999].

Corticosterone secretion by rat ZFR cells increased in response to steroidogenic precursors with different concentrations (10^{-7} M, 10^{-5} M, $P < 0.01$). The precursors include 25-hydroxy-cholesterol (25-OH-C), pregnenolone (Δ_5P), progesterone (P_4) and deoxycorticosterone (DOC). However these reactions were inhibited by addition of RUT (10^{-4} M) or EVO (10^{-4} M) respectively, ($P < 0.01$) (Figs. 3 and 4). This indicated that the last enzyme in biosynthesis of glucocorticoids, that is,11β-hydroxtlase was inhibited by Rut and EVO. However RUT and EVO did not show the ability to inhibit other enzymes in biosynthesis of glucocorticoids in this study.

The release of pregnenolone by ZFR cells of male rats increased with different concentrations of 25-OH-cholesterol (10^{-7} M, 10^{-5} M, $P < 0.01$). This reaction was not inhibited by addition of RUT (10^{-4} M) and EVO (10^{-4} M) respectively, (Fig. 5). This indicated that neither RUT nor EVO inhibited the activity of P450scc. However, the release of progesterone by ZFR cells of male rats increased with different concentrations of pregnenolone (10^{-7} M, 10^{-5} M, $P < 0.01$). This reaction was inhibited by addition of RUT (10^{-4} M) and EVO (10^{-4} M) respectively, ($P < 0.01$) (Fig. 6). This result indicated that both RUT and EVO inhibited the activity of 3β-HSD in ZFR cells of male rats.

Unlike cells that produce polypeptide hormones, which store large amounts of hormone in secretory vesicles ready for rapid release, steroidogenic cells store very little steroids. Thus a rapid steroidogenic response requires rapid synthesis of new steroids. The acute regulation of steroidogenesis (e.g., the rapid rise in serum cortisol following severe injury) is at the level of substrate access to P450scc, which is regulated at the level of cholesterol transport into the mitochondria [Miller, 1988; Stocco and Clark, 1996]. Orme-Johnson first showed that this acute steroidogenic response was accompanied by the rapid synthesis of a 37 kDa phosphoprotein [Pon and Orme-Johnson, 1986; Pon et al., 1986]. Stocco and Clark cloned this factor and named the steroidogenic acute regulatory protein, StAR [Clark et al., 1994]. StAR is principally expressed in the adrenal cortex and in the steroidogenic cells of the gonads. The relationship between StAR and EVO or RUT has not been reported. In this study, EVO (10^{-4} M) and RUT (10^{-4} M) does not influence the StAR protein expressions in male rat ZFR cell by 60 min incubation. However, both of them inhibits the StAR protein expression after 120 min incubation ($P < 0.05$).

A cell is said to be steroidogenic if it expresses the cholesterol side-chain cleavage enzyme, P450scc, which catalyzes the first and rate-limiting step in steroidogenesis. This mitochondrial enzyme catalyzes three distinct reactions, 20α-hydroxylation, 22-hydroxylation and scission of the 20,22 carbon–carbon bond, thus converting cholesterol to pregnenolon [Miller, 1988]. It is believed that P450scc is the only enzyme that can convert cholesterol to pregnenolone. The presence of P450scc renders a cell "steroidogenic" and able to make steroids de novo, as opposed to modifying steroids produced elsewhere, which occurs in many types of cells [Miller, 1988]. The relationship between P450scc and EVO or RUT has not been reported. In this study, EVO (10^{-4} M) and RUT (10^{-4} M) does not influence P450scc protein expressions in male rat ZFR cell after 120 min incubation.

These results suggest that EVO and RUT inhibit corticosterone production in rat ZFR cells via a mechanism involving: (1) a decreased activity of cAMP-related pathways; (2) a decreased activity of the steroidogenic enzymes, that is, 3β-hydroxysteroid dehydrogenase (3β-HSD) and 11β-hydroxylase (P450c11), during steroidogenesis; and (3) an inhibition of StAR protein expression. In conclusion, the present investigation demonstrated an inhibitory effect of EVO and RUT on the secretion of corticosterone by ZFR cells in male rats.

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