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Effects of *S*-petasin on cyclic AMP production and enzyme activity of P450scc in rat zona fasciculata-reticularis cells

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Abstract

Petasites hybridus is used in Chinese herbal medicine. *S*-petasin is a bioactive compound isolated from leaves or roots of *P. hybridus*, which has been used to relieve gastrointestinal pain, lung disease, and spasms of urogenital tract. We have demonstrated that *S*-petasin inhibited corticosterone release from rat zona fasiculata-reticularis cells. However, the mechanism and molecular effects of *S*-petasin on zona fasiculata-reticularis cells are still unclear. This study explored the effects of *S*-petasin on cellular adenosine 3′:5′-cyclic monophosphate (cAMP) production, the functions of steroidogenic enzymes including cytochrome P450 side-chain cleavage enzyme (P450scc), 11β-hydroxylase, and the expression levels of steroidogenic acute regulatory protein or P450scc. In this experiment, zona fasciculata-reticularis cells were incubated with *S*-petasin in the presence or absence of adrenocorticotropin (ACTH), 8-bromo-adenosine 3′:5′-cyclic monophosphate (8-Br-cAMP), forskolin, 25-OH-cholesterol, deoxycorticosterone at 37 °C for 0.5, 1 or 3 h. The media were used to measure the concentration of corticosterone or pregnenolone by radioimmunoassay. The cells were used to measure the content of cAMP by radioimmunoassay and extracted protein for Western blot or messenger RNA (mRNA) for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Our data demonstrated that (1) *S*-petasin inhibits ACTH- or forskolin-stimulated cellular cAMP production, (2) *S*-petasin increased the Michaelis constants of P450scc and 11β-hydroxylase and (3) *S*-petasin decreased the expression levels and mRNA of steroidogenic acute regulatory protein. In summary, the actions of *S*-petasin mediate the inhibition of cAMP formation, decrease the activities of key enzymes P450scc and 11β-hydroxylase, and reduce mRNA of steroidogenic acute regulatory protein and expression of steroidogenic acute regulatory protein.

Keywords: Zona fasciculata-reticularis cells; *S*-petasin; P450scc activity; 11β-Hydroxylase activity; Steroidogenic acute regulatory protein expression

1. Introduction

Petasites hybridus (L.) is used in Chinese herbal medicine. *S*-petasin is a bioactive compound isolated from *P. hybridus*. It has been shown that *S*-petasin possesses many biological effects including decrease of the severity tonsillitis, a spasmolytic activity of the gastrointestinal tract, and triggers asthmatic attacks (Debrunner and Neuenschwander, 1995). We previously reported that *S*-petasin inhibits the production of testosterone from rat testicular interstitial cells (Lin et al., 2000). We also have demonstrated that *S*-petasin decreased both basal and adrenocorticotropin (ACTH)-induced corticosterone release, and inhibited the release of corticosterone from rat zona fasciculata-reticularis cells via diminishing the activity of P450scc and 11β-hydroxylase (Chang et al., 2002). However, the mechanisms mediating the effects of *S*-petasin, and molecular and gene level interactions are still unclear.

The biosynthesis of all adrenal steroid hormones, in response to steroidogenic stimuli, begins with the cleavage of the side chain of cholesterol to form pregnenolone and ends with metabolizing deoxycorticosterone to form corticosterone. The first reaction is catalyzed by the cytochrome P450 side-chain cleavage enzyme (P450scc) (Simpson et al., 1979), and the last reaction is catalyzed by 11β-hydrox-

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ylase. It was interesting to know whether *S*-petasin affects the kinetic constants (V_{max} and Michaelis constant) of P450scc or 11β-hydroxylase to diminish its activity.

Various intracellular transduction mechanisms have been discovered. The most important event in the rat adrenal system is activation of adenylyl cyclase causing an increase in intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) concentration which activates protein kinase A (PKA) (Scheterson and McKnight, 1991). It was interesting to know whether *S*-petasin-associated signal transduction in adrenal cortical cells is mediated by affecting intracellular cAMP production to diminish ACTH-induced corticosterone secretion.

In the present study, we isolated zona fasciculata-reticularis cells from rat adrenals to investigate the involved mechanisms of *S*-petasin on corticosterone secretion. The approaches included the examination of the formation of the second messenger cAMP, the key enzyme ($P450_{SCC}$ and 11β-hydroxylase) activities, and the expression of rate-limiting elements, $P450_{SCC}$ and steroidogenic acute regulatory protein, at mRNA and protein levels. We hypothesize that the inhibitory role of *S*-petasin on corticosterone secretion is (1) in part related to the post-cAMP pathway, (2) affecting P450scc and 11β-hydroxylase activities, and (3) diminishing the protein and mRNA expressions of steroidogenic acute regulatory protein.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats weighing 300–350 g (2 months old) provided by National Yang-Ming University were housed in a temperature-controlled room (22 ± 1 °C) with 14 h of artificial illumination daily (0600–2000) and given food and water ad libitum. All animal experimentation has been conducted humanely and in conformance with policy statement of the Committee of National Yang-Ming University.

2.2. Preparation of zona fasciculata-reticularis cells for cell culture

Zona fasciculata-reticularis cells for culture were prepared following Purdy et al. (1991) procedures with minor modifications (Lo et al., 1998). Rat adrenal glands were excised, and then kept in an ice-cold 0.9% (w/v) NaCl solution. The adipose tissues were removed. The encapsulated glands were separated by forceps into capsule (mainly zona glomerulosa) and inner zone (mainly zona fasciculata-reticularis) fractions. The fractions of inner zone from 10–20 adrenals were assigned as one dispersion, then added to a polyethylene tube containing 1-ml Krebs–Ringer bicarbonate buffer with 3.6 mmol K^+/l, 11.1 mmol glucose/l and 0.2% bovine serum albumin (KRBGA medium) and collagenase 2 mg (Sigma, St. Louis, MO, USA). The tube was aerated with 95% O_2 and 5% CO_2, then incubated for 1 h at 37 °C in a shaker bath oscillating 100 cycles per minute. Generally, at least four dispersions ($n = 4$) of zona fasciculata-reticularis cells were incubated in each group. At the end of incubation, the zona fasciculata-reticularis cells were mechanically dispersed by repeated pipetting, then filtering through a nylon mesh. After centrifugation at 200 × *g* for 10 min, cells were washed with deionized water for disrupting red blood cells, and then the osmolarity was immediately restored with 10-fold Hanks balanced salt solution (HBSS). The cell number and cell viability (over 70%) were assessed by using a hemocytometer and the trypan blue exclusion method. The cells (5×10^4 cells/ml) were preincubated with KRBGA medium for 1 h at 37 °C in a shaker bath (50 cycles per minute) 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 30 min or 1 h. Post incubation and centrifugation, the medium was stored at −20 °C for corticosterone or pregnenolone radioimmunoassay. The cells were homogenized in 0.5 ml of 65% ice-cold ethanol by polytron (PT-3000, Kinematica, Luzern, Switzerland), and then centrifuged at 2000 × *g* for 10 min. The supernatants were lyophilized in a vacuum concentrator (Speed Vac, Savant, Holbrook, NY, USA), then reconstituted with assay buffer (0.05 M sodium acetate buffer with 0.01% azide, pH 6.2) before measuring the concentration of cAMP by radioimmunoassay.

2.3. Experiment: effects of S-petasin on corticosterone release by zona fasciculata-reticularis cells

The zona fasciculata-reticularis cells in 1 ml per tube of KRBGA medium were preincubated for 1 h at 37 °C under 95% O_2 and 5% CO_2. To determine the effect of *S*-petasin on adenyl cyclase, preincubated cells were incubated for 30 min with 0.3-ml medium containing ACTH (0 or 10^{-9} M), forskolin (0 or 10^{-4} M) or 8-Br-cAMP (0 or 10^{-4} M) combined with *S*-petasin (0 or 10^{-6}–10^{-5} M). After incubation, the media were stored at −20 °C for corticosterone analysis by radioimmunoassay.

To determine the effect of *S*-petasin on cellular cAMP production, preincubated cells were primed for 30 min with 0.5-ml medium in the presence or absence of 3-isobutyl-methyl-xanthine (IBMX, phosphodiesterse inhibitor, 1 mM) or IBMX combined with forskolin (10^{-4} M). After priming, cells were incubated for 30 min with 0.3-ml medium containing IBMX plus ACTH (0 or 10^{-9} M), or IBMX plus forskolin (0 or 10^{-4} M) combined with *S*-petasin (0 or 10^{-6}–10^{-5} M). After incubation, the cells were stored at −80 °C for intracellular cAMP analysis by radioimmunoassay.

To determine whether the toxicity of *S*-petasin has effect on corticosterone release, cells after preincubation were incubated for 1 h with 0.3-ml medium containing ACTH

(10^{-9} M) combined with *S*-petasin (0 or 10^{-6}–10^{-5} M). After incubation, the cells were washed and further incubated for 1 h with 0.3-ml medium containing ACTH (10^{-9} M). After this two-stage incubation, the media were stored at −20 °C for corticosterone analysis by radioimmunoassay.

2.4. Experiment: effects of S-petasin on biosynthesis of corticosterone

To measure the effect of *S*-petasin on P450scc activity, zona fasciculata-reticularis cells after preincubation were incubated for 1 h with or without *S*-petasin (10^{-5} M) in the presence or absence of 25-OH-cholesterol (10^{-6}–10^{-5} M, substrate for P450scc).

For kinetic analysis of *S*-petasin on P450scc activity, zona fasciculata-reticularis cells (5×10^4 cells/ml) were incubated for 1 h with trilostane (10^{-5} M) or trilostane plus 10^{-5} M *S*-petasin in the presence of 25-OH-cholesterol (10^{-7} to 10^{-3} M). For kinetic analysis of *S*-petasin on 11β-hydroxylase activity, cells after preincubation were incubated for 1 h with or without *S*-petasin (10^{-5} M) in the presence of deoxycorticosterone (10^{-8}–10^{-4}M, substrate for 11β-hydroxylase). After incubation and centrifugation at $200 \times g$ for 10 min, the supernatant was used to measure medium concentration of corticosterone, or pregnenolone by radioimmunoassay.

2.5. Western blot analysis

The Western blotting method has been reported previously (Kau et al., 1999a; Lo et al., 2000). The zona fasciculata-reticularis cells (1.5×10^6–3×10^6 cells) were incubated with medium containing *S*-petasin (0, 10^{-5}–10^{-4} M), or ACTH (10^{-8} M) for 3 h. At the end of incubation, cells were washed twice with ice-cold saline and dissolved with 50-μl lysis buffer. The lysis buffer consisted of 1.5% Na-Lauroylsacrosine, 2.5 mM Tris-base, 1 mM EDTA, 0.68% phenylmethylsulfonyl fluoride (PMSF), and 2% proteinase inhibitors, at pH 7.8. Cell mixtures were centrifuged for 10 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 sulphate (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 75 V for 15 min and then at 150 V for 40 min using a running buffer. The proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Perkin-Elmer Life Science Products, Boston, MA, USA) using a Trans-Blot SD semi-dry-transfer cell (170-3940, Bio-Rad, Hercules, CA, USA) at 60 mA (for 8 × 10-mm membrane) for 60 min in a transfer solution. The membranes were washed in buffer (TBS-T buffer, containing 0.8% NaCl, 0.02 M Tris-base, 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-steroidogenic acute regulatory protein antibody (1:1000, rabbit) or anti-P450scc antibody (1:2000, rabbit) in 5% nonfat dry milk of TBS-T buffer overnight at 4 °C. After four washes with TBS-T buffer that were 5 min each, the membranes were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG, 1:6000 dilution) in 5% nonfat dry milk of TBS-T buffer. The membranes were washed four times with TBS-T buffer, then the band for steroidogenic acute regulatory protein or P450scc was visualized by chemiluminescence (ECL Western blotting detection reagents, Amersham Pharmacia Biotech. Buckinghamshire, England). The chemiluminescence signal on X-ray film was scanned by a scanner (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA, USA) and quantification by the ImageQuaNT program (Molecular Dynamics). The P450scc and steroidogenic acute regulatory protein signals were corrected by β-actin signal.

2.6. Reverse transcriptase-polymerase chain reaction analysis

Zona fasciculata-reticularis cells (3×10^6–5×10^6 cells) were incubated with or without 10^{-4} M *S*-petasin for 30 min. Treatment with ACTH (10^{-8} M) was processed as a positive control. At the end of incubation, the cells were washed twice and total RNA was isolated with a TRIzol® reagent (5×10^6 cells/ml) (Molecular Research Center, Invitrogen, CA, USA). The procedures were conducted according to the manufacturer's instructions. RNA samples were dissolved in water containing 0.1% diethyl pyrocarbonate and quantified by measuring the absorbency at 260 nm. Aliquots containing 100 ng of RNA were assayed by the relative-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) procedure, which was modified from the method described by Ronen-Fuhrmann et al. (1998). Reverse transcriptase was conducted for 120 min at 37 °C using 250 ng of pd(T) primer and 50 units of Moloney murine leukemia virus RT (BioLabs, Beverly, MA, USA). The minus control of RT was processed to confirm that the mRNA samples were not contaminated by cellular DNA. PCR was performed in the presence of 1 μCi of [α-^{32}P]deoxy-ATP (3000 Ci/mmol), dNTPs (a mixture of dATP, dCTP, dGTP, and dTTP, 200 nM), and 500 ng of appropriate oligonucleotide primers. Oligonucleotide primers for the ribosomal protein L19 served as an internal control (Ronen-Fuhrmann et al., 1998). The number of cycles was examined to verify that the amplification was in the exponential phase. Following the PCR reaction, tracking dye was added to 10–40 μl of the PCR reaction mixture (100 μl) for analysis by 5% PAGE (Orly et al., 1994). The gels were dried and exposed to X-ray film and scanned with a scanner (Personal Densitometer; Molecular Dynamics). Quantification of scanned images was per-

formed according to the ImageQuant program (Molecular Dynamics). The radioactivity in each PCR band was normalized to the radioactivity of the L19 band.

The PCR oligonucleotide primer pairs were designed based on known cDNA sequences of the various target genes. The expected PCR products would be 246 base pairs (bp) for rat steroidogenic acute regulatory protein cDNA (Ronen-Fuhrmann et al., 1998), 536 bp for rat P450scc, and 194 bp for rat RPL19 (Orly et al., 1994). Forward (A, sense) and reverse (B, antisense) primers were as follows: rat P450scc A, 5′-AGAAGCTGGGCAACATGGAGTCAG-3′, rat P450scc B, 5′-TCACATCCCAGGCAGCTGCATGGT-3′, rat steroidogenic acute regulatory protein A, 5′-GCAGCAGGCAACCTGGTG-3′, rat steroidogenic acute regulatory protein B, 5′-TGATTGTCTTCGGCAGCC-3′ RPL19 A, 5′-CTGAAGGTCAAAGGGAATGTG-3′, RPL19 B, 5′-GGACAGAGTCTTGATGATCTC-3′.

2.7. Corticosterone radioimmunoassay

The concentrations of corticosterone (the major adrenal glucocorticoid in rats) in media were determined by radioimmunoassay as previously described (Chen et al., 1997; Lo et al., 1998). With this antiserum (PSW#4-9), the sensitivity of corticosterone radioimmunoassay was 5 pg per tube. The intra- and interassay coefficients of variation were 3.3% ($n=5$) and 9.2% ($n=4$), respectively.

2.8. cAMP radioimmunoassay

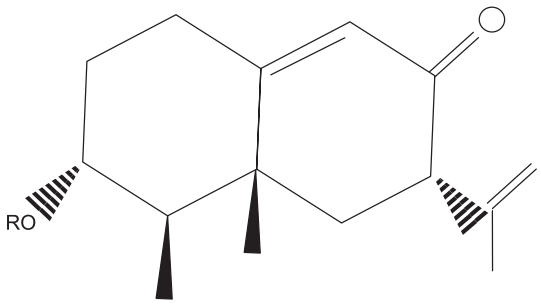
The intracellular levels of cAMP were measured by radioimmunoassay as described elsewhere (Lu et al., 1996; Chen et al., 1997; Lo et al., 1998). With anti-cAMP provided from Calbiochem-Novabiochem International (San Diego, CA, USA), the sensitivity of cAMP was 14 fmol per tube; the cross-reactivities were 0.0005% with cGMP, 0.02% with cIMP, and less than 0.0001% with cCMP, AMP, ADP, ATP, EDTA, and theophylline.

2.9. Pregnenolone radioimmunoassay

The concentration of pregnenolone in media was determined by radioimmunoassay as previously described (Kau et al., 1999b). The sensitivity of pregnenolone radioimmunoassay was 16 pg per tube. The intra- and interassay coefficients of variation were 2.3% ($n=6$) and 3.7% ($n=4$), respectively.

2.10. Materials

Bovine serum albumin, *N*-[2-hydroxyethyl]piperazine-*N*′[2-ethanesulphonic acid] (HEPES), Hanks balanced salt solution (HBSS), glucose, collagenase, adrenocorticotropin (ACTH), 3-Br-cyclic AMP (8-Br-cAMP), forskolin, 3-isobutyl-methyl-xanthine (IBMX), 25-hydroxy-cholesterol, phenylmethylsulfonyl fluoride (PMSF), and deoxycorticosterone were purchased from Sigma (St. Louis, MO, USA).



S-petasin ($C_{19}H_{26}O_3S$, MW=334)
R=COCH = CH(SMe) *cis*-

Fig. 1. Chemical structure of *S*-petasin.

Sodium dodecyl sulphate (SDS), bromophenol blue, and dithiothreitol were purchased from Research Organics (Cleveland, OH, USA). Proteinase inhibitor cocktail tablets were purchased from Boehringer Mannheim (Mannheim, Germany). Trilostane (4,5-epoxy-17-hydroxy-3-oxoandrostane-2-carbonitrile), an inhibitor of 3β-hydroxysteroid dehydrogenase (3β-HSD), was provided by Sanofi-Synthelabo (Malvern, PA, USA). *S*-petasin, whose structure given in Fig. 1, was kindly provided from National Research Institute of Chinese Medicine, Taipei, Taiwan, ROC. [^{3}H]Corticosterone and [^{3}H]pregnenolone were obtained from Amersham Life Science (Buckinghamshire, UK). The anti-pregnenolone antiserum was purchased from Biogenesis (Pool, England, UK). Anti-P450scc antibody was provided by Dr. Bon-Chu Chung (Hu et al., 1991), and anti-steroidogenic acute regulatory protein antibody was provided by Dr. D. M. Stocco (Lin et al., 1998). [α-^{32}P]Deoxy-ATP was obtained from NEN Life Science Products (Boston, MA, USA). The peroxidase-conjugated IgG fraction to mouse IgG and peroxidase-conjugated IgG fraction to rabbit IgG were purchased from ICN Pharmaceuticals (Aurora, OH, USA).

2.11. Statistical analysis

All values are given as the mean ± S.E.M. In some cases, the treatment means were test for homogeneity by one-way analysis of variance (ANOVA), and the difference between specific means was tested for significance by Duncan's multiple-range test (Steel and Torrie, 1960). In other cases, Student's *t* test was employed. A difference between two means was considered statistically significant when $P<0.05$.

3. Results

3.1. Effects of S-petasin on ACTH-, forskolin-, or 8-Br-cAMP-induced corticosterone release and intracellular cAMP production

Incubation of zona fasciculata-reticularis cells with ACTH (10^{-9} M), forskolin (10^{-4} M) or 8-Br-cAMP

(10^{-4} M) for 30 min increased corticosterone release ($P<0.01$, Fig. 2). *S*-petasin at 10^{-5} M significantly inhibited the ACTH, forskolin or 8-Br-cAMP-induced corticosterone release ($P<0.01$, Fig. 2). The *S*-petasin at 10^{-6} M only significantly diminished the forskolin induced corticosterone release ($P<0.05$, Fig. 2).

Incubation of zona fasciculata-reticularis cells with IBMX (1 mM) plus ACTH (10^{-9} M) or forskolin (10^{-4} M) for 30 min increased cellular cAMP production ($P<0.01$, Fig. 3). *S*-petasin at 10^{-6} or 10^{-5} M resulted in a decrease of ACTH- or forskolin-induced cellular cAMP production by zona fasciculata-reticularis cells ($P<0.05$ or $P<0.01$, Fig. 3).

3.2. Effect of S-petasin on corticosterone secretion

First incubation of zona fasciculata-reticularis with ACTH (10^{-9} M) combined with *S*-petasin (0 or 10^{-6}–10^{-5} M) for 1 h decreased corticosterone secretion ($P<0.05$ or $P<0.01$, Fig. 4). After the first incubation, the cells were washed, centrifuged, and further incubated with ACTH (10^{-9} M) for 1 h to test the effect of *S*-petasin on the viability of zona fasciculata-reticularis cells. The levels of corticosterone secretion after the second incubation were not significantly different (Fig. 4).

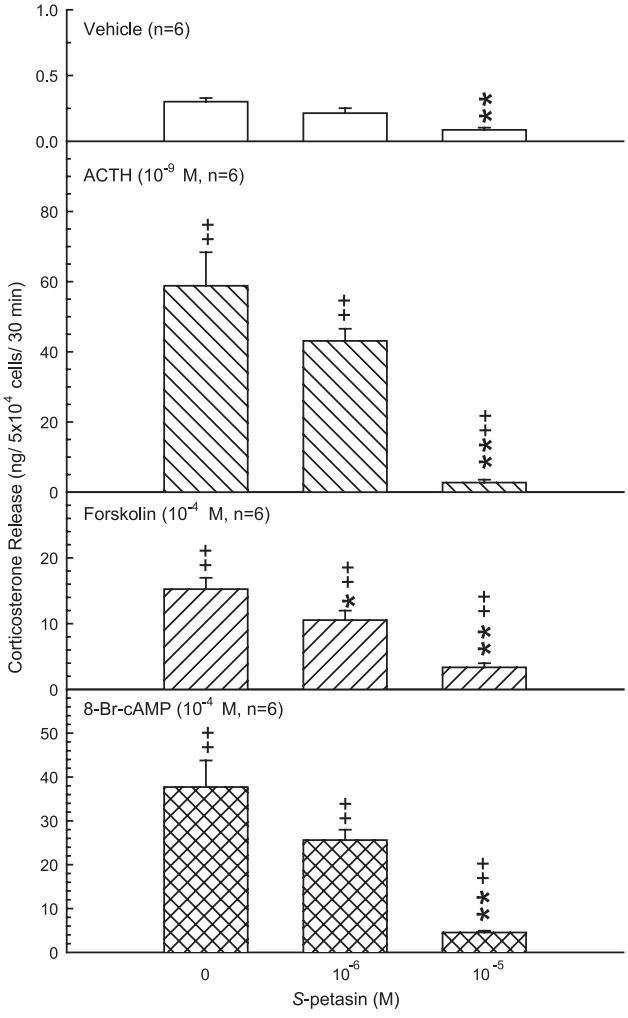


Fig. 2. Effects of *S*-petasin (10^{-6}–10^{-5} M) on the vehicle, ACTH (10^{-9} M)-, forskolin (10^{-4} M)-, and 8-Br-cAMP (10^{-4}) stimulated corticosterone release by zona fasciculata-reticularis cells from male rats. *$P<0.05$, **$P<0.01$ as compared with *S*-petasin = 0 M within respective group. $^{++}P<0.01$ as compared with vehicle-treated group. Each value represents mean ± S.E.M.

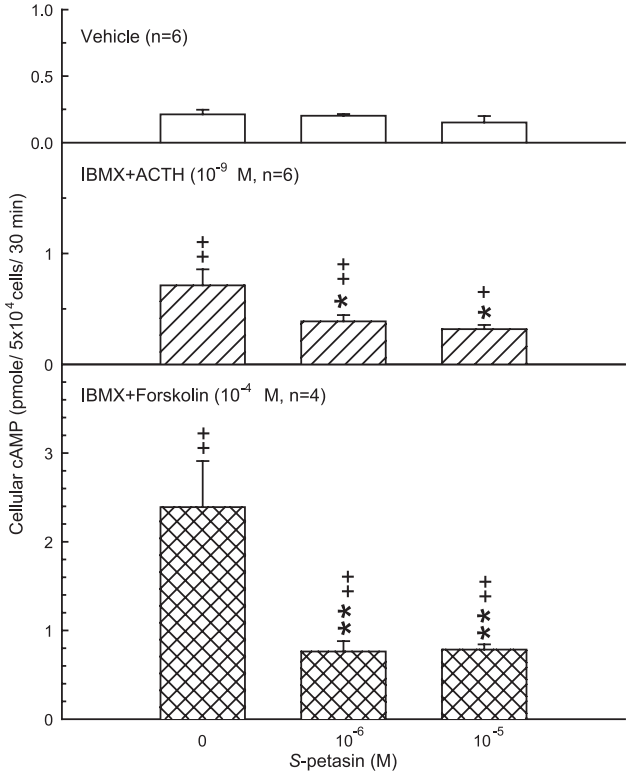


Fig. 3. Effects of *S*-petasin (10^{-6}–10^{-5} M) on the vehicle, IBMX + ACTH (10^{-9} M)-, and IBMX + forskolin (10^{-4} M)-stimulated cellular cAMP production by zona fasciculata-reticularis cells. *$P<0.05$, **$P<0.01$ as compared with *S*-petasin = 0 M within respective group. $^{+}P<0.05$, $^{++}P<0.01$ as compared with vehicle-treated group. Each value represents mean ± S.E.M.

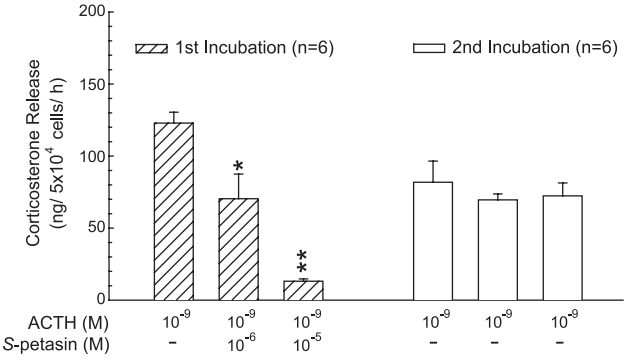


Fig. 4. The effect of *S*-petasin on corticosterone release by zona fasciculata-reticularis cells. *S*-petasin (10^{-6}–10^{-5} M) inhibited ACTH (10^{-9} M)-stimulated corticosterone release by zona fasciculata-reticularis cells after the first incubation. *$P<0.05$, **$P<0.01$ as compared with *S*-petasin = 0 M. The cells were challenged again with ACTH (10^{-9} M). There was no difference among three groups. Each value represents mean ± S.E.M.

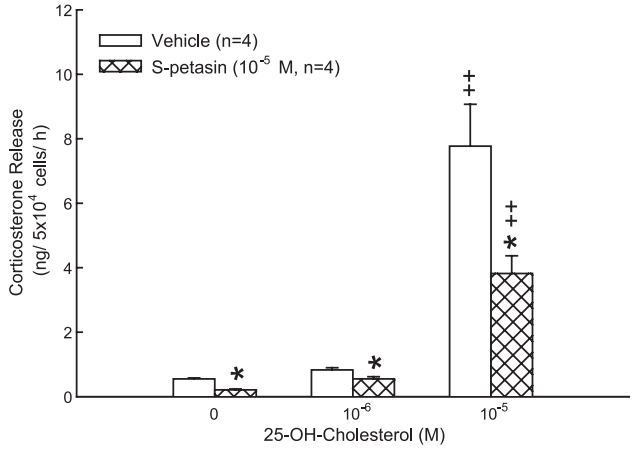


Fig. 5. Effects of *S*-petasin (10^{-5} M) on the activity of P450scc in zona fasciculata-reticularis cells. *$P<0.05$ as compared with *S*-petasin = 0 M within respective group. $^{++}P<0.01$ as compared with 25-OH-cholesterol = 0 M. Each value represents mean ± S.E.M.

3.3. Effects of S-petasin on biosynthesis pathway of corticosterone

To investigate the effects of *S*-petasin on P450scc, 25-OH-cholesterol (10^{-6}–10^{-5} M) were used to challenge the zona fasciculata-reticularis cells. The 25-OH-cholesterol at 10^{-5} M significantly increased corticosterone release by zona fasciculata-reticularis cells ($P<0.01$, Fig. 5). The *S*-petasin (10^{-5} M) inhibited basal and 25-OH-cholesterol (10^{-6}–10^{-5} M)-induced release of corticosterone ($P<0.05$, Fig. 5).

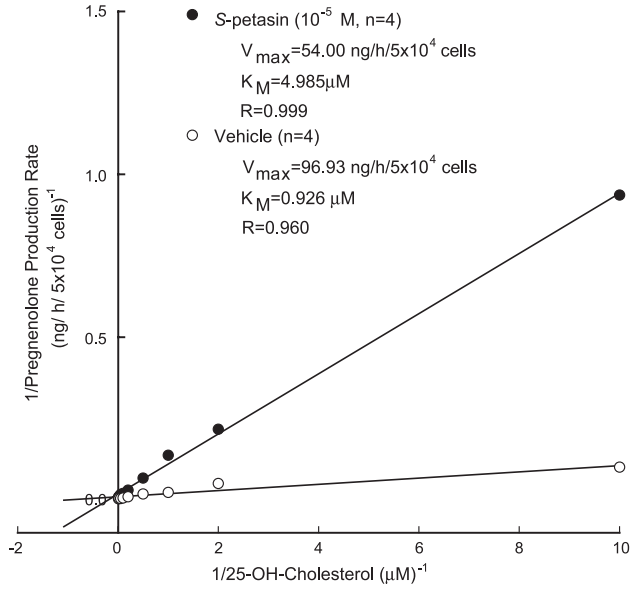


Fig. 6. Kinetic analysis of *S*-petasin inhibition on P450scc function. Double reciprocal plots of data were obtained from cultured zona fasciculata-reticularis cells challenged with 25-OH-cholesterol (10^{-7}–10^{-3} M). V_{max} for the *S*-petasin-treated group (54.00 ng/h/5 × 10^4 cells) was similar to the control group (96.93 ng/h/5 × 10^4 cells). K_M for the *S*-petasin-treated group (4.985 μM) was 4.6-fold greater than for the control group (0.926 μM).

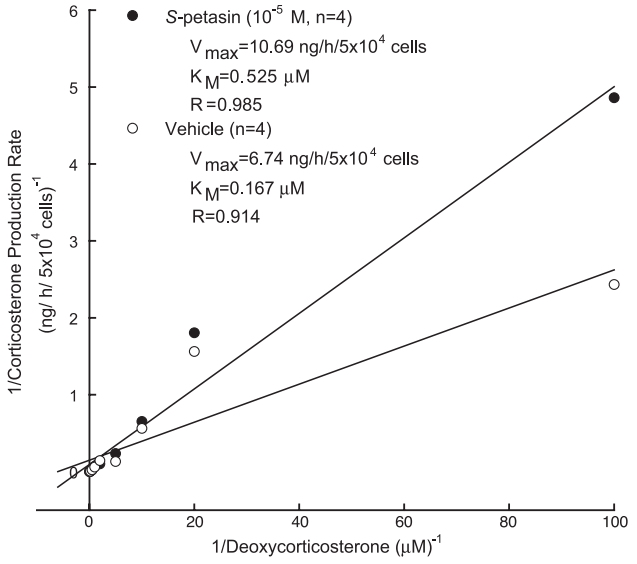


Fig. 7. Kinetic analysis of *S*-petasin inhibition on 11β-hydroxylase function. Double reciprocal plots of data were obtained from cultured zona fasciculata-reticularis cells challenged with deoxycorticosterone (10^{-8}–10^{-4} M). V_{max} for the *S*-petasin-treated group (10.69 ng/h/5 × 10^4 cells) was similar to the control group (6.74 ng/h/5 × 10^4 cells). K_M for the *S*-petasin-treated group (0.525 μM) was 3.1-fold greater than for the control group (0.167 μM).

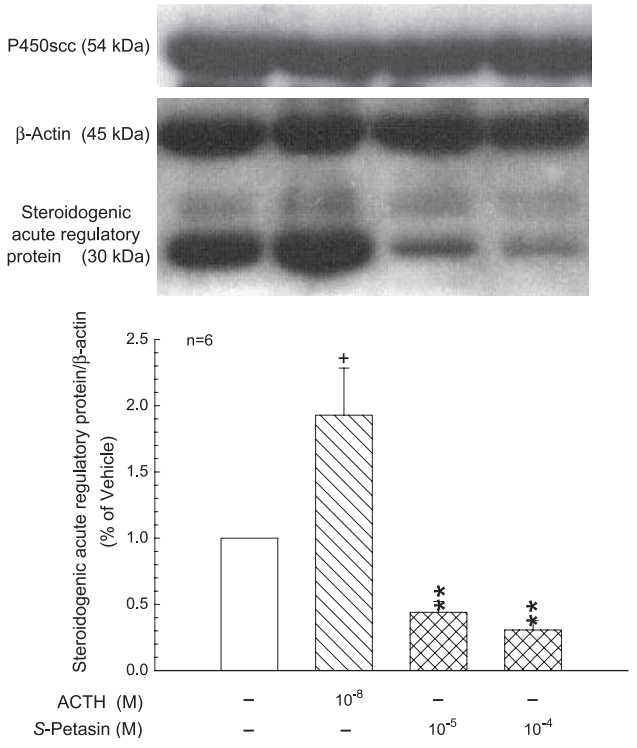


Fig. 8. P450scc and steroidogenic acute regulatory protein expression under ACTH (10^{-8} M), or *S*-petasin (10^{-5}–10^{-4} M) treatment. Western blot analysis of cell extracts subjected to SDS-PAGE and developed by enhanced chemiluminescence. P450scc (54 kDa) and steroidogenic acute regulatory protein (30 kDa) were detected by incubation with P450scc and steroidogenic acute regulatory protein antisera. This set of experiment was repeated several times with similar results. $^{+}P<0.05$ for ACTH effect. **$P<0.01$ for *S*-petasin effect. Each value represents mean ± S.E.M.

3.4. Effect of S-petasin on Michaelis constants of P450scc and 11β-hydroxylase

As shown in Fig. 6, *S*-petasin (10^{-5} M) decreased 25-OH-cholesterol (10^{-7}–10^{-3} M)-induced pregnenolone production from zona fasciculata-reticularis cells. The maximum velocities (V_{max}) were similar between the control group (96.93 ng/h/5 × 10^4 cells) and *S*-petasin group (54.00 ng/h/5 × 10^4 cells). The Michaelis constant (K_M) was 0.926 μM in the control group and 4.985 μM in the *S*-petasin group.

In Fig. 7, *S*-petasin (10^{-5} M) decreased deoxycorticosterone (10^{-8}–10^{-4} M)-induced corticosterone production from zona fasciculata-reticularis cells. The maximum velocities (V_{max}) were apparently similar between the control group (6.74 ng/h/5 × 10^4 cells) and *S*-petasin group (10.69 ng/h/5 × 10^4 cells). The Michaelis constant (K_M) was 0.167 μM in the control group and 0.525 μM in the *S*-petasin group.

3.5. Protein expression of steroidogenic acute regulatory protein and P450scc

Following 3-h incubation with zona fasciculata-reticularis cells, the resting amount of steroidogenic acute regulatory protein was significantly decreased by the admin istration of *S*-petasin (10^{-5}–10^{-4} M) ($P<0.01$, Fig. 8). After the administration of ACTH (10^{-8} M), the amount of steroidogenic acute regulatory protein was increased ($P<0.05$). There was no significant difference in P450scc protein expression among the control, ACTH, and *S*-petasin groups (Fig. 8).

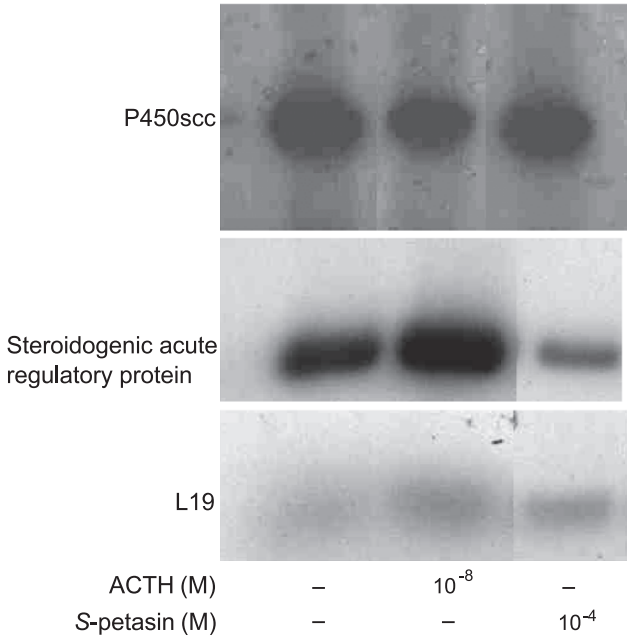


Fig. 9. P450scc and steroidogenic acute regulatory protein mRNA expression under ACTH (10^{-8} M), or *S*-petasin (10^{-4} M) treatment. After rat zona fasciculata-reticularis cells were challenged with ACTH or *S*-petasin for 30 min, RNA was extracted, and 100 ng of RNA was assayed by relative-quantitative RT-PCR. Ribosomal L19 served as an internal control. This set of experiment was repeated three times with similar results.

3.6. Messenger RNA expression of steroidogenic acute regulatory protein and P450scc

The objective of this experiment was to investigate steroidogenic acute regulatory protein and P450scc mRNA expression in rat zona fasciculata-reticularis cells when challenged with ACTH (10^{-8} M) or *S*-petasin (10^{-4} M) for 30 min. In our results, L19 was not affected by in vitro treatment. The mRNA of steroidogenic acute regulatory protein in the zona fasciculata-reticularis cells was increased by ACTH and decreased by *S*-petasin (Fig. 9). There was no significant change in P450scc mRNA level among the control, ACTH and *S*-petasin groups (Fig. 9).

4. Discussion

ACTH is the major hormone which regulates not only acute glucocorticoid secretion but also the expression and maintenance of adrenal cell specific functions. The specific functions include: its receptor number (Penhoat et al., 1994), the expression of the genes encoding the steroidogenic enzymes (Simpson and Waterman, 1988), cAMP second messenger systems which activate cAMP-dependent protein kinases (PKA) (Clegg et al., 1992; Schimmer, 1980; Wong et al., 1992). In this study, ACTH increased corticosterone secretion (Fig. 2), cellular cAMP production (Fig. 3), expression levels of steroidogenic acute regulatory protein (Fig. 8) and mRNA of steroidogenic acute regulatory protein in zona fasciculata-reticularis cells (Fig. 9).

Previously we have reported that *S*-petasin inhibited ACTH-, forskolin-, or 8-Br-cAMP-induced corticosterone release from rat zona fasciculata-reticularis cells (Chang et al., 2002). In this study, we showed that *S*-petasin inhibited not only ACTH-, forskolin-, or 8-Br-cAMP-induced corticosterone release (Fig. 2), but also ACTH-, forskolin-stimulated cellular cAMP production (Fig. 3). These results suggested that one of the cascade pathways of *S*-petasin was beyond the membrane receptor to directly intervene with the formation of cAMP.

In the present study, administration of *S*-petasin for either 1/2 or 1 h inhibited ACTH-induced corticosterone release (Figs. 2 and 4). After removing (or withdrawing) *S*-petasin, the levels of corticosterone release from zona fasciculata-reticularis cells in response to ACTH challenge following the second incubation were similar to the control level (without pretreatment of *S*-petasin). These results suggested that the inhibition of corticosterone secretion by *S*-petasin was independent of its toxicity to zona fasciculata-reticularis cells.

In order to understand the mechanism by which *S*-petasin inhibits P450scc function, the zona fasciculata-reticularis cells were challenged with serial doses of 25-

OH-cholesterol combined with trilostane (an inhibitor of 3β-HSD). The kinetic analysis of 25-OH-cholesterol treated zona fasciculata-reticularis cells revealed that P450scc had an apparent K_M of 0.926 μM and a V_{max} of 96.93 ng/h/5×10^4 cells. The V_{max} of the *S*-petasin group was similar to the control group, but the K_M was 4.985 μM, which was almost 4.6-fold of the control value. The parameters of the same V_{max} with different K_M suggested a competitive inhibition mechanism (Fig. 6). It indicated that *S*-petasin might interfere with the formation of the binding complex of P450scc and cholesterol.

In order to investigate the mechanism by which *S*-petasin inhibits 11β-hydroxylase function, the zona fasciculata-reticularis cells were challenged with serial doses of deoxycorticosterone (a substrate for 11β-hydroxylase). The kinetic analysis of deoxycorticosterone treated zona fasciculata-reticularis cells revealed that 11β-hydroxylase had an apparent K_M of 0.167 μM and a V_{max} of 6.74 ng/h/5×10^4 cells. The V_{max} of the *S*-petasin group was similar to the control group, but the K_M was 0.525 μM, which was almost 3.1-fold of the control value. These characters were consistent with a competitive inhibition mechanism (Fig. 7). It indicated that *S*-petasin might interfere with the formation of the binding complex of 11β-hydroxylase and deoxycorticosterone.

The rate-limiting step in steroid biosynthesis is the conversion of cholesterol to pregnenolone by P450scc. In addition to this important enzyme, another protein, steroidogenic acute regulatory protein, has been identified as being involved in the acute regulation of steroid production in steroidogenic tissues. The steroidogenic acute regulatory protein represents a most possible candidate for the transfer of cholesterol from cellular stores to the inner mitochondrial membrane. Based on data obtained from the regulated expression of steroidogenic acute regulatory protein and the observed accompanying increase in steroid biosynthesis (Clark et al., 1994; Stocco and Clark, 1996), we propose that steroidogenic acute regulatory protein is rapidly synthesized in the cytosol in response to hormone stimulation. In the present study, the protein and mRNA expressions of both P450scc and steroidogenic acute regulatory protein were examined by Western blot and RT-PCR assay. Based on Western blot and RT-PCR data, no significant differences were observed in P450scc protein and mRNA expressions among the control, ACTH and *S*-petasin groups after zona fasciculata-reticularis cells were incubated with ACTH or *S*-petasin (Figs. 8 and 9). However, *S*-petasin could inhibit steroidogenic acute regulatory protein and mRNA expressions (Figs. 8 and 9). Since *S*-petasin decreases the corticosterone production, this suggests that the acute inhibitory effects of *S*-petasin on corticosterone secretion may be an inhibition of P450scc function rather than an effect on P450scc protein and mRNA expressions, and an inhibition of steroidogenic acute regulatory protein and mRNA expressions.

In summary, the present results demonstrated that *S*-petasin could inhibit corticosterone secretion via cascade of inhibitions, i.e. reduction of cAMP production and the post-cAMP pathway. The post-cAMP pathway involved diminishing P450scc and 11β-hydroxylase functions. The diminishing P450scc function was due to the diminishing steroidogenic acute regulatory protein and mRNA expressions.

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