Effects of *S*-Petasin on Corticosterone Release in Rats

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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 *Petasites hybridus*. *S*-petasin has been used to relieve gastrointestinal pain, lung disease, and spasms of the urogenital tract. However, the side effect of *S*-petasin on endocrine systems are still not clear. This study explored the effects of *S*-petasin on the release of corticosterone in vivo and in vitro. An intravenous injection of *S*-petasin (10 μg/kg) decreased both basal and adrenocorticotropin (ACTH)-induced plasma corticosterone concentration in male rats. In vitro, *S*-petasin (3×10^{-6}-10^{-4} M) caused a significant reduction of basal and ACTH-stimulated release of corticosterone from the enzymatically dispersed rat zona fasciculata-reticularis (ZFR) cells in a dose-dependent manner. In order to study possible mechanisms, ZFR cells were incubated with *S*-petasin (10^{-5} M) in the presence or absence of forskolin (adenylate cyclase activator, 10^{-6}-10^{-4} M), 8-Br-cAMP (a cAMP analogue, 10^{-6}-10^{-4} M), 25-OH-cholesterol (pregnenolone biosynthesis precursor, 10^{-5} M) combined with trilostane (a blocker of 3β-hydroxysteriod dehydrogenase, 3β-HSD, 10^{-6} M) and deoxycorticosterone (corticosterone biosynthesis precursor, 10^{-9}-10^{-6} M) at 37°C for 1h. The concentration of pregnenolone and corticosterone in media were measured by radioimmunoassay. The stimulatory effects of corticosterone secretion induced by forskolin (10^{-5}-10^{-4} M), 8-Br-cAMP (10^{-5}-10^{-4} M) and deoxycorticosterone (10^{-7}-10^{-6} M) were reduced by *S*-petasin at 10^{-5} M. The stimulatory effects of pregnenolone secretion induced by 25-OH-cholesterol combined with or without trilostane was reduced by *S*-petasin at 10^{-5} M. These results suggest that *S*-petasin inhibits the production of corticosterone from rat ZFR cells in part through decreasing the activities of adenylyl cyclase, P450scc and 11β-hydroxylase.**

Key Words: corticosterone, *S*-petasin, rat, zona fasciculata-reticularis cells

Introduction

Petasites hybridus (L.) is used in Chinese herbal medicine. *S*-petasin is a bioactive compound isolated from *Petasites hybfidus*. It has been shown that *S*-petasin possesses many biological effects including decreasing the severity tonsillitis, a spasmolytic activity of the gastrointestinal tract, and triggers asthmatic attacks (3). We have reported that *S*-petasin inhibits the production of testosterone from rat testicular interstitial cells (5). However, the effects of *S*-petasin on the adrenal system is still unclear. This is the first study of experiments to explore the effects of *S*-petasin on the secretion of corticosterone, the effects of *S*-petasin on the biosynthesis pathways of corticosterone, the mechanisms mediating the effects of *S*-petasin, and molecular and gene level interactions.

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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)(12), and the last reaction is catalyzed by 11β-hydroxylase. It was interesting to know whether *S*-petasin affects P450scc or 11β-hydroxylase activity.

Various intracellular transduction mechanisms have been discovered. The most important event in the rat adrenal system activates of adenylate cyclase causing an increase in intracellular cyclic adenosine 3',5'-monophosphate (cAMP) concentration which activates protein kinase A (PKA)(10). It was interesting to know whether *S*-petasin signal transduction in adrenal cortical cells is mediated by affecting adenylate cyclase activity.

The present study examined : [1] the effects of a single intravenous injection of *S*-petasin on both basal and adrenocorticotropin (ACTH)-induced plasma concentration of corticosterone in male rats, and [2] the direct effects of *S*-petasin on the secretion of corticosterone by rat zona fasciculata-reticularis (ZFR) cells to explore their possible mechanisms.

Materials and Methods

Animals

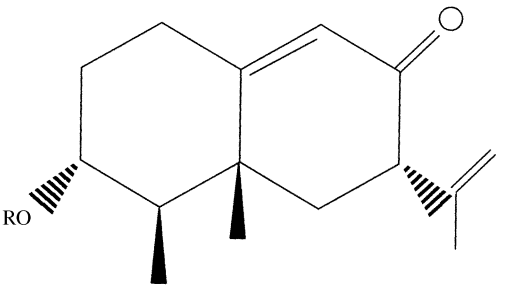
Male Sprague-Dawley rats weighing 300-350g (2-month old) provided by National Yang-Ming University were housed in a temperature-controlled room (22 ± 1 °C) with 14 h of artificial illumination daily (06 h - 20 h) and given food and water *ad libitum.*

Materials

Bovine serum albumin (BSA), N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid (HEPES), Hank's balanced salt solution (HBSS), glucose, collagenase, adrenocorticotropin (ACTH), 3-Br-cyclic AMP, forskolin, 25-hydroxy-cholesterol, trilostane and deoxycorticosterone were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). S-petasin whose structure given in Fig. 1 were kindly provided by Dr. Yun-Lian Lin from National Research Institute of Chinese Medicine, Taipei, Taiwan. [^{3}H]-corticosterone and [^{3}H]-pregnenolone were obtained from Amersham International Plc. (Bucks, U.K.)

In Vivo Experiment

Male rats were anaesthetized with ether and then catheterized via the right jugular vein (15,17). Twenty hours later, conscious rats were injected intravenously with vehicle (saline, 1 ml/kg), *S*-petasin (10 μg/kg), ACTH (5 μg/kg), or ACTH (5 μg/kg) combined with *S*-petasin (10 μg/kg) *via* the jugular catheter. Blood samples (0.5 ml each) were collected at 0, 0.5 h, 1 h, 2 h, 3 h and 4 h after treatment. Plasma was separated by centrifugation at 10,000 × *g* for 1 min. The concentration of corticosterone in each plasma sample was measured by radioimmunoassay (RIA) after ether extraction.



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

Fig. 1. Structure and molecular weight of *S*-petasin.

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

ZFR cells for culture were prepared following Purdy *et al.* (9) procedures with minor modifications (6). Rat adrenal glands were excised, then kept in an ice-cold 0.9% (w/v) NaCl solution, and adipose tissue was 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 5-6 adrenals were assigned as one dispersion, then added to a polyethlene tube containing 1 ml Krebs-Ringer bicarbonate buffer with 3.6 mmol K^+/l, 11.1 mmol glucose/l and 0.2% BSA (KRBGA medium, pH 7.4) 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. At the end of incubation, the ZFR cells were mechanically dispersed by repeated pipetting, then filtered through a nylon mesh. After centrifugation at 200 × *g* for 10 min, cells were washed with deionized water to disrupt red blood cells, then the osmolarity was immediately restored with 10-fold Hank's balanced salt solution (HBSS, pH 7.4). After centrifugation at 200 × *g* for 10 min, the supernatant was discarded, and the pellets were resuspended in 3 ml of KRBGA medium. An aliquot (20 μl) was used to count the number of cells in a

hemocytometer after staining with 0.05% nigrasin stain. The viability of isolated cells was 70-75%. Cells for cultures were further diluted to a concentration of 5×10^4 cells/ml and divided into the test tubes.

Effects of S-Petasin on Corticosterone Secretion

The ZFR cells in 1 ml/tube KRBGA medium were preincubated for 1 h at 37°C under 95% O_2 and 5% CO_2. To studying *S*-petasin effects on corticosterone production, cells after preincubation were incubated for 1 h with 0.3 ml medium containing ACTH (10^{-9} M), combined with or without *S*-petasin(10^{-6}-10^{-4} M). To determine the effect of *S*-petasin on adenylate cyclase or cAMP, cells after preincubation were incubated for 1 h with 0.3 ml medium containing forskolin (0 or 10^{-6}-10^{-4} M) or 8-Br-cAMP (0 or 10^{-6}-10^{-4} M) combined with *S*-petasin (10^{-5} M).

Effects of S-Petasin on Biosynthesis of Corticosterone

To measure the effect of *S*-petasin on P450scc activity, ZFR ells 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^{-5} M, precursor of P450scc) combined with trilostane (10^{-6} M). To measure the *S*-petasin effect on 11β-hydroxlase activity, cells after preincubation were incubated for 1 h with or without *S*-petasin (10^{-5} M) in the presence or absence of deoxycorticosterone (10^{-9}-10^{-6} M, precursor of 11β-hydroxlase activity). After incubation and centrifugation at $200 \times g$ for 10 minutes, the supernatant was used to measure medium concentration of corticosterone, or pregnenolone by RIA.

RIA of Corticosterone

The concentration of corticosterone in plasma and media was determined by RIA as described previously (1,6). With this antiserum (PSW#4-9), a RIA was established for the measurement of plasma corticosterone levels. The sensitivity of the corticosterone RIA was 5 pg/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 described previously (4). The antipregnenolone antiserum was purchased from Biogenesis Inc (Sandown, NH, USA). The sensitivity of pregnenolone RIA was 16 pg/tube. The intra- and interassay coefficients of variation were 2.3% (n=6) and 3.7% (n=4), respectively.

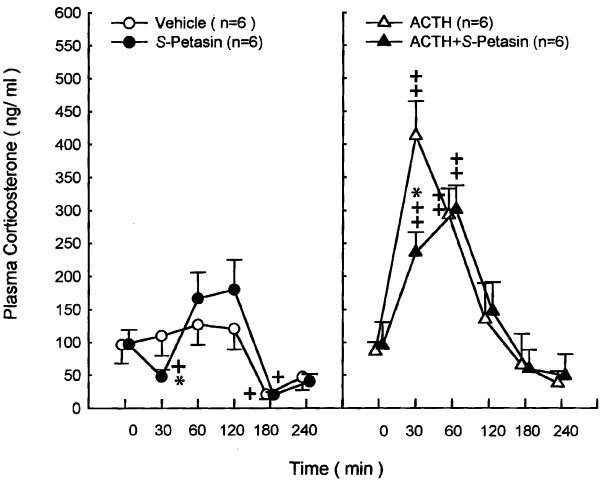


Fig. 2. Effect of a single intravenously injection of *S*-petasin on the concentration of plasma corticosterone (left panel), and that of *S*-petasin on the response of plasma corticosterone to ACTH (right panel). Male rats were intravenously injected with saline, *S*-petasin (10 μg/kg), ACTH (5 μg/kg), ACTH plus *S*-petasin via the right jugular vein. $^*P < 0.05$ as compared between control and experimental group at 30 minutes. $^+P < 0.05$, and $^{++}P < 0.01$ as compared with the value at 0 min within each group, respectively. Each value represents the mean ± SEM.

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 (14). A difference between two means was considered statistically significant when *P* was less than 0.05.

Results

Effects of S-Petasin on Plasma Corticosterone Concentration

Basal levels of plasma corticosterone in vehicle and *S*-petasin-challenged groups were 96.69±28.85 ng/ml (n=8) and 97.17 ± 22.32 ng/ml (n=8), respectively. At 30 min after injection of *S*-petasin (10 μg/kg), the concentration of corticosterone was reduced (109.93 ± 30.42 ng/ml in vehicle group vs. 47.70 ± 10.79 ng/ml in *S*-petasin group, $P < 0.05$) (Fig. 2). No difference was observed ($P \geq 0.05$) between the control and experimental groups on corticosterone concentration at 60, 120, 180, and 240 minutes after treatment (Fig. 2).

Thirty min after injection of ACTH (5 μg/kg), the concentration of plasma corticosterone increased

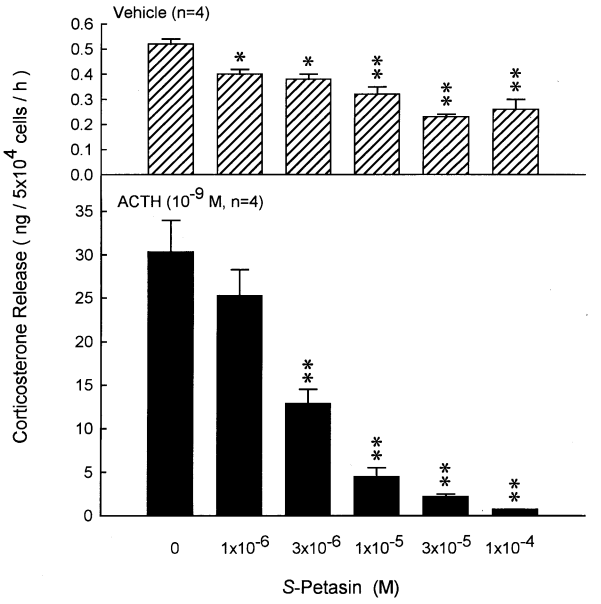


Fig. 3. Effects of S-petasin (10^{-6}-10^{-4} M) on the vehicle, and ACTH (10^{-9} M)- stimulated corticosterone release by ZFR cells in male rats. *P < 0.05, and **P < 0.01 as compared with *S*-petasin =0 M within respective group. Each value represents mean ± SEM.

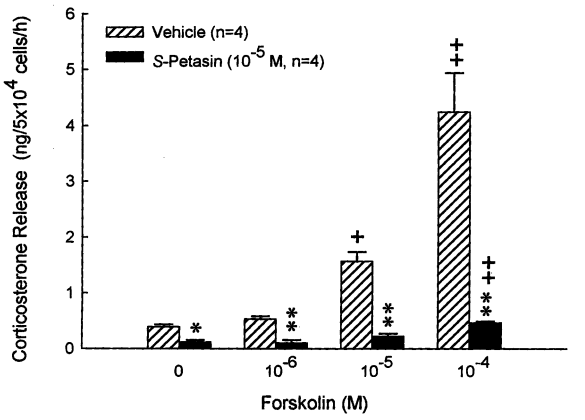


Fig. 4. Forskolin (10^{-6}-10^{-4} M)-induced release of corticosterone decreased in the presence of *S*-petasin (10^{-5} M) with ZFR cells. *P < 0.05, and **P < 0.01 as compared with vehicle group within each forskolin concentration. ^{+}P < 0.05, and ^{++}P < 0.01 as compared with forskolin=0 M in respective to control or experimental group. Each value represents mean ± SEM.

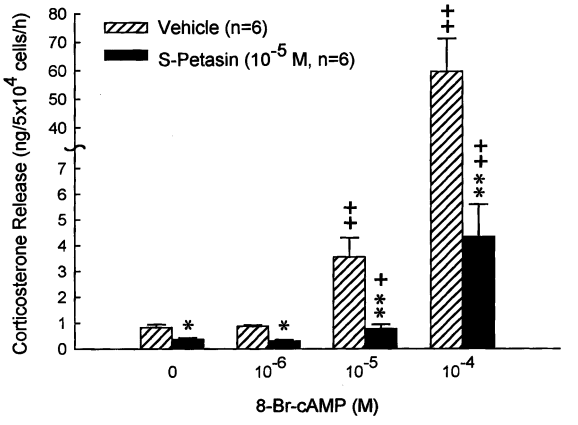


Fig. 5. The 8-Br-cAMP(10^{-6}-10^{-4} M)-induced corticosterone release from ZFR cells diminished in the presence of *S*-petasin (10^{-5} M). *P < 0.05, and **P < 0.01 as compared with vehicle group within each 8-Br-cAMP concentration. ^{+}P < 0.05, and ^{++}P < 0.01 as compared with 8-Br-cAMP =0 M in respective to either control or experimental group. Each value represents mean ± SEM.

by 480% (P < 0.01) and was maintained for 1 h. However, the group treated with ACTH (5 μg/kg) and *S*-petasin (10 μg/kg) reduced (P < 0.05) of the ACTH-stimulated release of corticosterone (412.54 ± 52.50 ng/ml in ACTH group vs. 235.69 ± 30.91 ng/ml in ACTH+*S*-petasin group; Fig. 2). No inhibitory effect of *S*-petasin on plasma corticosterone levels induced by ACTH was observed ($P \geq 0.05$) after 30 min. (Fig. 2).

Effects of S-Petasin on ACTH-, Forskolin-, or 8-Br-cAMP-Induced Corticosterone Release in Vitro

When compared with controls, the doses of *S*-petasin in the range of 10^{-6}M-10^{-4} M caused a dose-dependent inhibition of corticosterone release from ZFR cells (P < 0.05 or P < 0.01) (Fig. 3). Incubation of ZFR cells with ACTH (10^{-9} M) for 1 h increased corticosterone release (P < 0.01). ACTH with *S*-petasin (3×10^{-6}-10^{-4} M) resulted in an inhibition of ACTH-stimulated release of corticosterone (P < 0.01).

Forskolin (10^{-5}-10^{-4} M) increased (P < 0.05) corticosterone release in a dose-dependent pattern (Fig. 4). Combination of forskolin (10^{-6}-10^{-4} M) with *S*-petasin (10^{-5} M) resulted in a diminution of forskolin-induced corticosterone release by ZFR cells (Fig. 4).

Corticosterone release by ZFR cells was also increased (P < 0.05) by 8-Br-cAMP (10^{-5}-10^{-4} M) in a dose-dependent phenomenon (Fig. 5). The 8-Br-cAMP (10^{-6}-10^{-4} M) combined with *S*-petasin (10^{-5} M) decreased 8-Br-cAMP-induced release of corticosterone (P < 0.05; Fig. 5).

Effects of S-Petasin on Biosynthesis Pathway of Corticosterone

The 25-OH-cholesterol (10^{-5} M), trilostane (10^{-6} M) or 25-OH-cholesterol combined with trilostane increased pregnenolone release by ZFR cells (P < 0.01) (Fig. 6). *S*-petasin (10^{-5} M) inhibited pregnenolone release by ZFR cells in the vehicle or 25-OH-cholesterol combined with trilostane treatments (P < 0.05 and P < 0.01, Fig. 6).

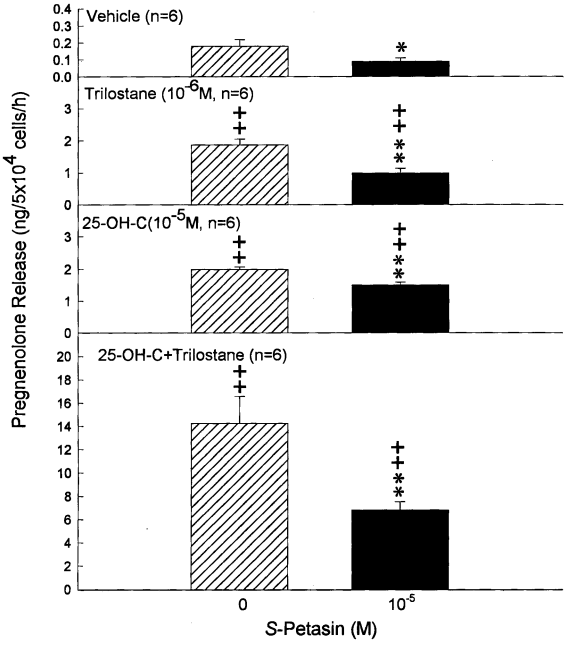


Fig. 6. Effects of *S*-petasin (10^{-5} M) on the vehicle, and 25-OH-cholesterol (10^{-5} M)- induced release of pregnenolone from rat ZFR cells in the presence of trilostane (10^{-6} M). $^{*}P<0.05$, and $^{**}P < 0.01$ as compared with *S*-petasin=0 M within each treatment (vehicle, trilostane,25-OH-cholesterol,25-OH-cholesterol+trilostane). $^{+}P < 0.05$, and $^{++}P < 0.01$ as compared with vehicle group within each *S*-petasin group (0 or 10^{-5} M). Each value represents mean ± SEM.

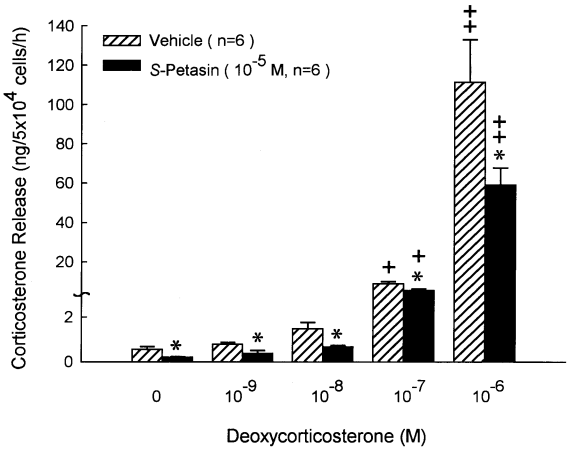


Fig. 7. Effects of *S*-petasin (10^{-5} M) on the activity of 11β-hydroxylase in ZFR cells from male rats. $^{*}P < 0.05$ as compared with vehicle group within each deoxycorticosterone concentration. $^{+}P < 0.05$, and $^{++}P < 0.01$ as compared with deoxycorticosterone =0 M in respective to vehicle or *S*-petasin group. Each value represents mean ± SEM.

Deoxycorticosterone (10^{-7}-10^{-6} M) increased corticosterone release by ZFR cells ($P<0.05$ and $P < 0.01$) (Fig. 7). The *S*-petasin (10^{-5} M) inhibited deoxycorticosterone (10^{-9}-10^{-6} M)-induced release of corticosterone ($P < 0.05$, Fig. 7).

Discussion

ACTH is the major hormone which regulates not only acute glucocorticoid secretion, but also the expression and maintenance of adrenal cell specific functions, i.e. ACTH receptor number (8), which is in agreement with those obtained in the mouse and human adrenocortical cell lines (7). Thus, the increased steroidogenic responsiveness following ACTH treatment is due not only to the regulatory effects of ACTH on the expression of the genes encoding the steroidogenic enzymes (13), but also, due to the effects of ACTH on the expression of its own receptors. Most, if not all, of the actions of ACTH on the adrenal cortex are believed to be mediated through the cAMP second messenger system with the activation of cAMP-dependent protein kinases (PKAs) (2, 11, 18). In this study, physiological concentration of ACTH increased the corticosterone release.

From the *in vivo* study, the inhibitory effect of *S*-petasin was a short-term phenomenon. For both basal and ACTH-stimulated situation, the decrease in corticosterone concentration was observed only at 30 minutes after *S*-petasin (Fig. 2). Both the basal and the ACTH-stimulated groups had a decrease in plasma corticosterone concentration after 180 minutes. The differences in corticosterone concentration between time 0 and at time 180 and 240 might suggest an initial stress response in the rats. From Figure 3, the dose-dependent inhibition of corticosterone release was significant within the study range between 10^{-6}-10^{-4} M. The selected concentration at 10^{-5} M was in the sensitivity range of *S*-petasin's effect.

It is known that forskolin increases cAMP production by activating adenylate cyclase (16). The mechanism of forskolin's positive inotropic effect is related to a cAMP-dependent increase in Na^{+} permeability which results in an indirect augmentation of calcium release (19). In this study, forskolin increased corticosterone release by ZFR cells (Fig. 4) consistent with the actions of ACTH mediated through cAMP (2,11,18). The inhibition of forskolin effect by *S*-petasin indicates its mechanism is beyond the membrane receptor level.

The 8-Br-cAMP increased release of corticosterone by ZFR cells (Fig. 5) is consistent with the cAMP cascade as the 2nd messenger of ACTH (2, 11,18). However, *S*-petasin inhibited 8-Br-cAMP-induced corticosterone release suggests the inhibition by *S*-petasin is beyond the membrane receptor level and beyond the cAMP production.

The synthesis of corticosterone needs four key enzymes including P450scc, 3β-HSD, 21-hydroxylase and 11β-hydroxylase to convert cholesterol to corticosterone. Pregnenolone release should increase

when concentration of 25-OH-cholesterol was increased (Fig. 6). The inhibition of pregnenolone release by *S*-petasin suggests that *S*-petasin has a mechanism that interferes with P450scc enzyme activities. To inhibit 3β-HSD activity, trilostane decreases the utilization of substrate to become product (progesterone). It resulted in the accumulation of substrate as shown in Fig. 6. After inhibiting enzyme activity, by trilostane, the release of substrate was still lower in the *S*-petasin group. This may suggest that the production of pregnenolone was diminished, i.e. inhibiting the P450scc system. These data suggest the activity of P450scc was blocked by *S*-petasin. Supplementation of both 25-OH-cholesterol and trilostane resulted in a synergistic accumulation of pregnenolone release (Fig. 6). *S*-petasin showed a very significant inhibition of the pregnenolone release as expected (Fig. 6).

Deoxycorticosterone is metabolized to corticosterone by 11β-hydroxylase. Supplementation of the medium with more substrate (deoxycorticosterone) should produce more product (corticosterone) as shown in Fig. 7. *S*-petasin inhibited corticosterone release at all concentrations tested. This suggests that *S*-petasin also inhibits the activity of 11β-hydroxylase.

In summary, this study demonstrated that [1] *S*-petasin decreased both basal and ACTH-induced plasma corticosterone secretion, and [2] *S*-petasin inhibited the secretion of corticosterone from rat ZFR cells via diminishing the activity of P450scc and 11β-hydroxylase.

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