Effects of Adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf.) Hull Extracts on the Secretion of Progesterone and Estradiol *In Vivo* and *In Vitro*

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Adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf.) has been used as a traditional Chinese medicine for dysfunction of the endocrine system. However, there have been few studies on the effects of adlay seed on the endocrine system. In the present study, both the *in vivo* and *in vitro* effects of methanolic extracts of adlay hull (AHM) on progesterone synthesis were studied. AHM was partitioned with four different solvents: water, 1-butanol, ethyl acetate, and *n*-hexane. Four fractions, namely, AHM-Wa (water fraction), AHM-Bu (1-butanol fraction), AHM-EA (ethyl acetate fraction), and AHM-Hex (*n*-hexane fraction), were respectively obtained. Granulosa cells (GCs) were prepared from pregnant mare serum gonadotropin-primed immature female rats and were challenged with different reagents, including human chorionic gonadotropin (hCG; 0.5 IU/ml), 8-bromo-adenosine-3′,5′-cyclic monophosphate (8-Br-cAMP; 0.1 m*M*), forskolin (10 μ*M*), 25-OH-cholesterol (10 μ*M*), and pregnenolone (10 μ*M*), in the presence or absence of AHM (100 μg/ml). The functions of steroidogenic enzymes, including protein expression of the steroidogenic acute regulatory protein (StAR), cytochrome P450 side chain cleavage enzyme (P450scc), protein kinase A (PKA), and aromatase activity, were investigated. The expression of StAR mRNA was also explored by using real-time reverse transcription–polymerase chain reaction. In the *in vivo* study, AHM decreased plasma progesterone and estradiol levels after an intravenous injection of AHM (2 mg/ml/kg). In the *in vitro* studies, AHM decreased progesterone and estradiol *via* inhibition of (i) the cAMP-PKA signal transduction pathway, (ii) cAMP accumulation, (iii) P450scc and 3β-HSD enzyme activities, (iv) PKA, P450scc and StAR protein expressions and StAR mRNA expression, and (v) aromatase activity in rat GCs. These results suggest that AHM decreased the production of progesterone *via* mechanisms involving the inhibition of the cAMP pathway, enzyme activities, and the protein expressions of P450scc and StAR in rat GCs. Exp Biol Med 232:1181–1194, 2007

Key words: adlay hulls; progesterone; estradiol; aromatase; P450scc; StAR

Introduction

Coix lachryma-jobi L. var. *ma-yuen* Stapf. (a Chinese medicinal plant named Yi-yi-lan), commonly called adlay (Job's tears), is an annual crop. It has long been consumed as both an herbal medicine and a food supplement. From ancient times, adlay has been used in Asian countries for the treatment of rheumatism, warts, neuralgia, and the female endocrine system. It has been described in the ancient Chinese medical book Pen-Taso Kang Mu (1) as an efficient remedy for a number of maladies and as being particularly beneficial for the digestive system. It is widely planted in Taiwan, China, and Japan, and it is considered to be a healthy food supplement.

Recent studies have demonstrated some of the physio-

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logic effects of adlay extracts. Adlay extracts inhibit the growth of Ehrlich ascites sarcoma, and their active components have been identified as coixenolides (2). A number of benzoxazinones have been isolated from adlay seeds and demonstrated to exhibit anti-inflammatory activity (3). The coixans A, B, and C isolated from adlay seed express hypoglycemic activity in rats (4). The lipid components in plasma and feces decrease in rats fed with adlay seed (5). The ingestion of coix seed tablets increases the activities of cytotoxic T lymphocytes and natural killer cells (6). A decrease in fibrinolytic activities in plasma has been observed in rats on an adlay seed mixed diet (7). Numerous reports have indicated that the consumption of adlay seeds is beneficial to human health (3, 8–13). In addition, adlay has long been used in the folk medicine of Chinese as a nourishing food to regulate the female endocrine system (1).

However, different extractions of adlay seed have been demonstrated to exhibit different effects. For example, a methanolic extract of adlay seed suppressed human lung cancer cell COX-2 gene activity (13). The methanol (MeOH) extract also exhibited antiproliferative and chemopreventive effects on mouse lung cancer both *in vitro* and *in vivo* (14). Dehulled adlay modulates the immune response of T helper 1 and 2 cells (12). A water extract of adlay seed has been demonstrated to increase COX-2, ERK 1/2, and PKC-alpha expressions, which induce embryotoxicity and enhance uterine contractility during pregnancy in rats (15).

Although adlay has many biologic functions, its action on the endocrine system has not yet been studied extensively. One study indicated that adlay bran extract suppresses progesterone biosynthesis in rat granulosa cells (GCs; Ref. 16). Another study indicated that adlay hull extract suppresses corticosterone release from rat zona fasciculate-reticularis cells (17). Therefore, it is conceivable that adlay plays an important role in regulating endocrine functions. Nevertheless, some medical reports have also suggested that adlay seeds should not be consumed during pregnancy, although little supporting scientific evidence was provided (18). Therefore, the effects of adlay seeds on pregnancy remain unclear and deserve further examination.

Progesterone and estradiol are pregnancy-related ovarian hormones that are responsible for preparing the reproductive tract for zygote implantation and the subsequent maintenance of the pregnant state (19). Whether adlay affects the secretion of progesterone and estradiol in GCs is still unknown. Recently, we have been constructing different primary cell culture model systems in order to study the effects of different Chinese medicines on the endocrine system. We have found that some traditional Chinese medicines, such as bufalin, Chansu, digoxin, ginsenoside-Rb1, and evodiamine, exhibit inhibitory effects on peripheral hormone production (20–24). The inhibition caused by these Chinese medicines might occur *via* the action on different specific sites in rat GCs, Leydig cells, and the anterior pituitary.

The purpose of this study was to examine the direct effect of adlay extracts on the production of steroid hormones (progesterone [P_4] or estradiol [E_2]) *in vivo* and *in vitro*. We examined whether adlay extracts exert direct effects on the production of progesterone and estradiol *in vivo*. We also examined whether methanolic extracts of adlay hull (AHM) exert direct effects on the production of the cAMP–protein kinase A (PKA) pathway or on the function of the cytochrome P450 side chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), or aromatase in rat GCs. We also quantified the chemical compounds that could affect the production of progesterone or estradiol in rat GCs. Figure 1 shows a simple schematic depicting the steroid biosynthetic pathway with the enzymes involved in estradiol and progesterone synthesis.

Materials and Methods

Reagents. Pregnant mare serum gonadotropin (PMSG), Dulbecco's modified Eagle medium (DMEM)/F12, fatty acid–free bovine serum albumin (BSA), penicillin G, sodium bicarbonate, streptomycin sulfate, human chorionic gonadotropin (hCG), insulin, medium-199 (M199), L-glutamine, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES), P_4, BSA, glucose, 8-Br-cyclic AMP (8-Br-cAMP), forskolin (FSK), phenylmethylsulfonyl fluoride (PMSF), 25-hydroxy cholesterol (25-OH-cholesterol), pregnenolone, and dimethyl sulfoxide (DMSO) were purchased from the Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulphate (SDS), bromophenol blue, and dithiothreitol were purchased from Research Organics Inc. (Cleveland, OH). 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-HSD, was provided by Sanofi-Synthelabo Inc. (Malvern, PA). We obtained ^{3}H-androstenedione, ^{3}H-pregnenolone, and ^{3}H-progesterone from Amersham Life Science Limited (Buckinghamshire, UK). The antipregnenolone antiserum was purchased from Biogenesis Inc. (Sandown, NH). A cAMP enzyme immunoassay (EIA) system was obtained from Assay Designs Inc. (Ann Arbor, MI).

Plant Materials. Adlay was purchased from a local farmer who planted the Taichung Shuenyu No. 4 (TSC4) variety of *Coix lachryma-jobi* L. var. *ma-yuen* Stapf. in Taichung, Taiwan, in March 2000 and harvested it in July of the same year. The air-dried adlay seed was separated into the following three different parts: adlay hull, adlay testa, and polished adlay. All of the materials were blended in powder form and screened through a 20-mesh sieve (aperture: 0.94 mm).

Methanol Extractions of Adlay Seeds. Each sample powder (100 g) was extracted with 1 liter of methanol stirred on a Thermolyn Nuova stirring/heating plate (Dubuque, IA) at room temperature for 24 hrs. The

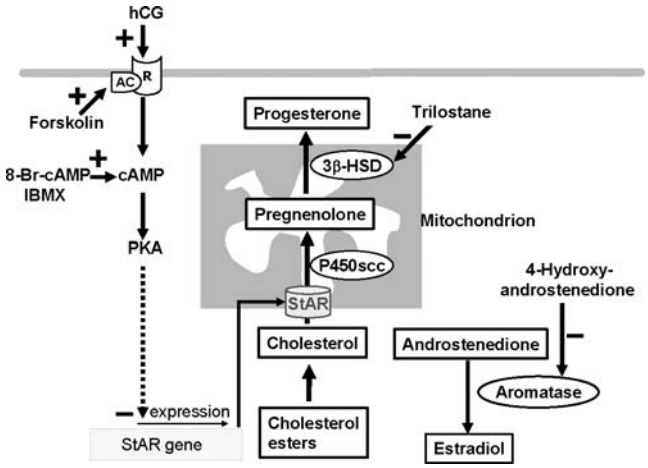

Figure 1. Simple scheme of the steroid biosynthetic pathway with enzyme for progesterone and estradiol production in rat granulosa cells. R, receptor; AC, adenylyl cyclase; PKA, protein kinase A; + indicates that the hormone or drug (hCG, forskolin, 8-Br-cAMP) could enhance this step; – indicates that drugs (trilostane, 4-hydroxyandrostenedione) could inhibit this step.

contents were filtered through no. 1 filter paper (Whatman Inc., Hillsboro, OR). The filtrate was concentrated to dryness under vacuum conditions in order to obtain a dried methanolic extract, and was stored at –20°C. The methanolic extracts from different parts of the adlay seed were named as AHM (hull), ATM (testa), and PAM (polished adlay).

Partition and Fractionation of Methanol Extracts of AHM. As an initial step to identify and characterize medicinally relevant chemical compounds present in adlay hulls, we performed liquid extraction using solvents of increasing polarity. Since adlay hulls contain myriad active and nonactive compounds located in different parts of the plant cell, we used solvents of different polarity to solvate the compound and quantified active compounds present in adlay hull. Figure 2A shows the scheme for the preparation of antiprogesterone or antiestradiol extracts from adlay hulls. The sample powder (7.3 kg) was extracted three times with 22 liters of methanol at room temperature for 2 weeks (5 days for each extraction). In order to minimize methanol consumption in the large-scale adlay hull methanol extractions, we prolonged the extraction time instead of using additional methanol. The plant material was filtered off, and the methanolic extracts were combined and concentrated under reduced pressure by a rotary vacuum evaporator. The obtained dry extract (AHM, 74.5 g) was suspended in 750 ml H_2O, followed by an extraction with same the volumes of *n*-hexane, ethyl acetate, or 1-butanol, yielding four fractions denoted as AHM-Hex, 19.05 g (*n*-hexane fraction); AHM-EA, 11.1 g (ethyl acetate fraction); AHM-Bu, 9.98 g (1-butanol fraction); and AHM-Wa, 20.35 g (water fraction). The fraction with the highest antiprogesterone and antiestradiol activity (AHM-EA) was chromatographed on a silica gel column using a Hex/EA/acetone/MeOH gradient system to yield 13 subfractions: A (100% hexane), B (5% EA/Hex), C (10% EA/Hex), D (20% EA/Hex), E (40% EA/Hex), F (60% EA/Hex), G (80% EA/Hex), H (100% EA), I (25% acetone/EA), J (50% acetone/EA), K (100% acetone), L (50% MeOH/acetone), and M (100% MeOH). The effects of the subfractions of AHM-EA (AHM-EA-A~AHM-EA-M) on progesterone and estradiol release by rat GCs were further studied. For the *in vitro* treatment of rat GCs, adlay extract powder was dissolved in DMSO to prepare a stock solution. The final concentration of DMSO was less than 0.1%.

High-Performance Liquid Chromatography (HPLC) Analysis of AHM-EA Fractions F and G. The components of the adlay extracts were quantified using a previously described method (25) with slight modifications. The most active fractions, F and G, were subjected to HPLC analysis using a KH_2PO_4:CH_3CN mobile system. A Cosmosil 5C18-MS column (5 μm, 25 cm × 4.6 mm) was used to quantify the components in AHM-EA-F and AHM-EA-G (Fig. 2B). The quantification results revealed that AHM-EA-F contained quercetin, naringenin, and gallic acid, and that the AHM-EA-G contained naringenin, quercetin, vanillin, syringaldehyde, gallic acid, syringic acid, and *p*-coumaric acid.

Isolation and Culture of Rat GCs. Immature female Sprague-Dawley rats, 25~27 days of age, were housed in a temperature-controlled room (22°C ± 1°C) with 14 hrs of artificial illumination daily (0600 to 2000 hrs), and were given food and water *ad libitum*. The preparation of GCs was modified from methods described elsewhere (26, 27). The immature female rats were injected subcutaneously with PMSG (15 IU/rat). At 48 hrs later, the rats were killed by cervical dislocation. Ovaries were excised and transferred into the sterile DMEM/F12 (1:1) medium, which contained 0.1% BSA, 20 mM HEPES, 100 IU/ml penicillin G, and 50 μg/ml streptomycin sulfate. The 5–10 ovaries were assigned as a single dispersion. After trimming free fat and connective tissues, the follicles were punctured with a 26-gauge needle in order to release the GCs. The harvested cells were pelleted and resuspended in growth medium (DMEM/F12 containing 10% fetal calf serum, 2 μg/ml insulin, 100 IU/ml penicillin G, and 100 μg/ml streptomycin sulfate). Cell viability was greater than 90%, as determined using a hemocytometer and the trypan blue method. The GCs were aliquoted into 24-well plates at approximately 1×10^5 cells per well and were incubated at 37°C with 5% CO_2/95% air for 2 days. Generally, at least six dispersions ($n = 6$) of the GCs were incubated in each group. Morphologically, the cultured GCs maintained a characteristic round (or polygonal) shape, throughout our culture conditions.

Gel Electrophoresis and Western Blotting. The Western blotting method has been reported previously (28, 29). The GCs (2×10^6 cells) were incubated with medium containing AHM-EA-G (0 or 100 μg/ml) for 2 hrs. At the end of the incubation, the cells were washed twice with ice-cold saline and detached by trypsinization (1.25 mg/ml). The cells were collected and extracted in homogenization buffer

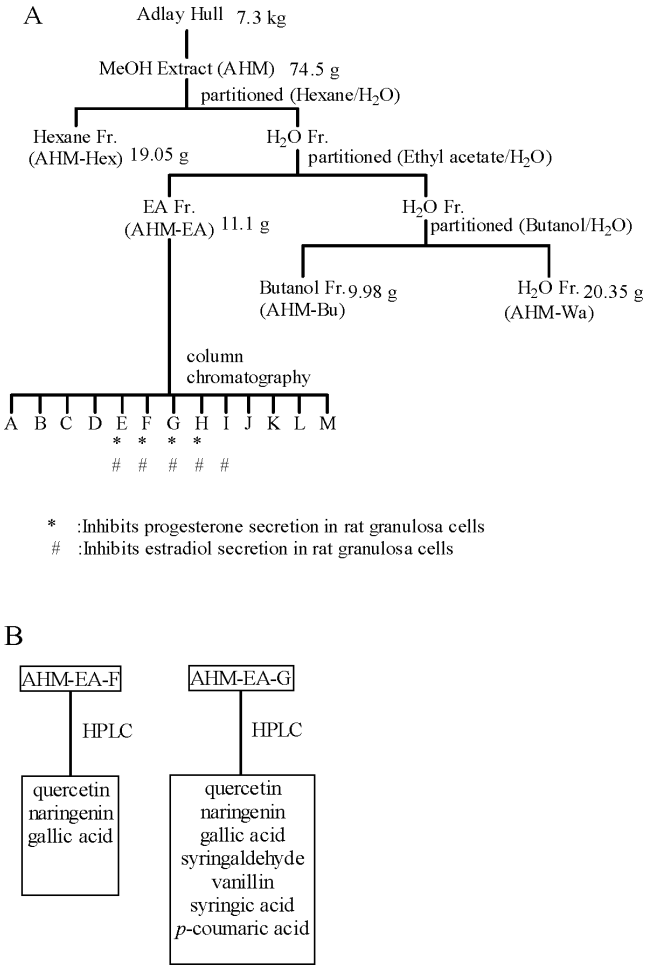

Figure 2. Scheme for extraction, partition, and fractionation of antiprogesterone and antiestradiol fractions from adlay hulls (A). Flow chart of the quantification of components from AHM-EA-F and AHM-EA-G (B). Fractions (Fr.) E to H exhibited the most potent inhibitory activity against progesterone secretion. Fractions E to I exhibited the most potent inhibitory activity against estradiol secretion.*Each of the AHM-EA subfractions E, F, G, and H at 100 μg/ml inhibited progesterone release in rat GCs compared with the subfraction at 0 μg/ml ($n = 6$, $P < 0.05$; Duncan's multiple-range test). #Each of the AHM-EA subfractions E, F, G, H, and I at 100 μg/ml inhibited estradiol release in rat GCs compared with the subfraction at 0 μg/ml ($n = 6$, $P < 0.05$; Duncan's multiple-range test; preliminary data not shown).

(pH 8.0) containing 1.5% Na-lauroylsarcosine, 1×10^{-3} *M* EDTA, 2.5×10^{-3} *M* Tris base, 0.68% PMSF, and 2% proteinase inhibitor cocktail, and then disrupted by ultrasonic sonication in an ice bath. The cell extracts were centrifuged at 13,500 *g* for 10 mins (29). The supernatant fluid was collected and the protein concentration determined by the Bradford colorimetric method (30). Extracted proteins were denatured by boiling for 5 mins in SDS buffer (0.125 *M* Tris base, 4% SDS, 0.001% bromophenol blue, 12% sucrose, and 0.15 *M* dithiothreitol; Ref. 31). The proteins (20 g) in the samples were separated on 12% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gels at 75 V for 15 mins and then at 150 V for 40 mins using a running buffer. The proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (NEN Life Science Products Inc., Boston, MA) using a Trans-Blot SD semi-dry transfer cell (170-3940; Bio-Rad, Hercules, CA) at 64 mA (for an 8 × 10 mm membrane) for 45 mins in a blotting solution. The membranes were washed in TBS-T buffer (0.8% NaCl, 0.02 *M* Tris base, and 0.3% Tween-20, pH 7.6) for 5 mins and then blocked by 120-min incubation in blocking buffer (TBS-T buffer containing 5% nonfat dry milk). Then the membranes were incubated with a mixture of anti-P450scc antibodies (1:2000), anti-StAR protein antibodies (1:1000), and β-actin antibodies (1:2000) in 5% nonfat dry milk of TBS-T buffer overnight at 4°C. The other membranes were incubated with anti-PKA protein antibodies (1:1000) in 5% nonfat dry milk of TBS-T buffer overnight at 4°C, and after one wash for 15 mins and three washes for 5 mins each time with TBS-T buffer, the membranes were incubated for 1 hr with horseradish peroxidase–conjugated goat anti-rabbit IgG (1:6000 dilution) or horseradish peroxidase–conjugated goat anti-mouse IgG (1:8000 dilution) in 5% nonfat dry milk of TBS-T buffer. The membranes were washed four times with TBS-T buffer, and then the bands for P450scc, StAR, β-actin, and PKA were visualized by chemiluminescence (ECL; Western blotting detection reagents; Amersham International, Buckinghamshire, UK).

Real-Time Reverse Transcription–Polymerase Chain Reaction (RT-PCR) Analysis. The same source of total RNA used to define gene expression profiles was used in real-time RT-PCR experiments following the instructions outlined in the LightCycler-RNA amplification kit SYBR Green I manual (Roche Molecular Biochemicals, Mannheim, Germany). Synthesis of cDNA was carried out in a LightCycler (Roche Molecular Biochemicals) in a capillary as follows: 20 μl reaction mix containing 500 ng DNase I–treated total RNA, 4 μl LightCycler–RT-PCR reaction mix SYBR Green I (final concentration: 1×), 5 m*M* $MgCl_2$, 0.4 μl LightCycler–RT-PCR enzyme mix, and 5.0 pmol forward and reverse primers for both genes. For reverse transcription, the reaction was incubated at 55°C for 30 mins and at 95°C for 30 secs. For PCR, the reaction was incubated at 95°C for 10 secs, 55°C for 5 secs, and 72°C for 10 secs. The total number of reaction cycles was 50. The expected sizes for the PCR products were 246 bp for the rat StAR cDNA; 536 bp for the rat P450scc, and 194 bp for the rat RPL19. Real-time RT-PCR assays were conducted in duplicate for each sample, and the mean value was used for calculation of expression levels. Finally, the P450scc and the StAR expressions were calculated in relation to RPL19 expression by the RelQuant Relative Quantification software Version 1.01 (Roche Molecular Biochemicals).

The forward (A, sense) and the reverse (B, antisense) primers were: Rat P450scc A, 5′-AGAAGCTGGGCAA CATGGAGTCAG-3′; Rat P450scc B, 5′-TCACATCC CAGGCAGCTGCATGGT-3′; Rat StAR A, 5′-GCAG CAGGCAACCTGGTG-3′; Rat StAR B, 5′-TGATTGTCTTCGGCAGCC-3′; RPL 19 A, 5′-

CTGAAGGTCAAAGGGAATGTG-3′; and RPL 19 B, 5′-GGACAGAGTCTTGATGATCTC-3′.

Effects of Adlay Extracts on Progesterone and Estradiol Release *In Vivo*. Diestrous or estrous rats were catheterized *via* the right jugular vein (32, 33). Twenty hours later, they were injected with saline (1 ml/kg), AHM-EA-G (2 mg/ml/kg), hCG (5 IU/ml/kg), or hCG plus AHM-EA-G *via* the jugular catheter. Blood samples (0.5 ml each) were collected at 0, 15, 30, 120, 180, 240, and 360 mins after the challenge. The plasma was separated by the centrifugation of blood samples at 10,000 *g* for 1 min. The concentration of progesterone and estradiol in the plasma was measured by radioimmunoassay (RIA).

Effects of Adlay Extracts on *In Vitro* Progesterone and Estradiol Release by Rat GCs. To study the effects of adlay extracts on the secretion of progesterone in rat GCs, the GCs were aliquoted into 24-well plates at approximately 1×10^5 cells per well, incubated at 37°C with 5% CO_2/95% air for 2 days, and then incubated with 1 ml BSA-MED-199 medium containing adlay extracts (0, 0.1, 1, 10, and 100 μg/ml) in the presence of vehicle (0.1% DMSO), hCG (0.5 IU/ml), forskolin (10^{-5} *M*), or 8-Br-cAMP (10^{-4} *M*) for 120 mins. The media were collected and the concentrations of progesterone were measured by RIA.

To study the accumulation of cAMP in response to adlay extracts, the rat GCs were preincubated with 10^{-3} *M* of the phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine (IBMX) and adlay extracts in the presence or absence of hCG (0.5 IU/ml) for 1 hr. At end of the incubation, the cells were homogenized in 0.5 ml 65% ice-cold ethanol using a polytron homogenizer (PT-3000; Kinematica AG, Luzern, Switzerland) and then centrifuged at 2000 *g* for 10 mins. The supernatants were lyophilized in a vacuum concentrator (Speed Vac; Savant, Holbrook, NY), then reconstituted with assay buffer (0.05 *M* sodium acetate buffer containing 0.01% azide, pH 6.2) before measuring the concentration of cAMP by EIA.

To study the influence of adlay extracts on the early enzymatic steps of steroidogenesis, the GCs were aliquoted into 24-well plates at approximately 1×10^5 cells per well, incubated at 37°C with 5% CO_2/95% air for 2 days, and then incubated with 1 ml BSA-MED-199 medium containing adlay extracts (0 and 100 μg/ml) in the presence of vehicle (0.1% DMSO) or precursors of steroidogenesis, such as 25-OH-cholesterol (10^{-5} *M*), pregnenolone (10^{-5} *M*), or the 3β-HSD inhibitor trilostane (10^{-5} *M*), for 120 mins. Progesterone or pregnenolone levels in media were measured by RIA.

To study the effects of adlay extracts on the secretion of estradiol in rat GCs, the androgen substrate androstenedione (20 μg/ml) was added to the BSA-MED-199 medium containing adlay hull extracts (0, 1, 10, and 100 μg/ml) in the presence of vehicle (0.1% DMSO) for 120 mins. The media were collected, and the concentrations of estradiol were measured by RIA. All adlay extracts were dissolved in DMSO.

Effects of Subfractions or Compounds From AHM on Aromatase Activity by Rat GCs. For studying the effects of adlay extracts on the aromatase activity in rat GCs, the aromatase activity was measured by use of the tritiated water release assay according to the protocol of Lephart and Simpson (34), with slight modifications. The rat GCs (1×10^5 cells) were incubated with medium containing ^{3}H-androstenedione (100 n*M*, 0.2 μCi) in the presence of vehicle (0.1% DMSO), AHM-EA-F (100 μg/ml), AHM-EA-G (100 μg/ml), narinigenin (50 and 100 μg/ml), 4-hydroxyandrostenedione (4-OHA, an aromatase inhibitor; 50 μ*M*), or forskolin (10^{-5} *M*) for 24 hrs. The media were collected, and the tritiated water released from each sample was extracted and its activity determined by liquid scintillation. Aromatase activity was expressed as picomoles of androstenedione converted per 24 hrs per 1×10^5 cells.

Hormone and cAMP Assays. The concentration of progesterone in the medium was determined by RIA, as described elsewhere (35, 36). Using antiprogesterone serum no. W5, the sensitivity of the progesterone RIA was 5 pg per assay tube. The intraassay and interassay coefficients of variation (CVs) were 4.8% ($n = 5$) and 9.5% ($n = 4$), respectively.

The concentration of pregnenolone in the medium was determined by RIA, as described elsewhere (22, 37). Using antipregnenolone serum, the sensitivity of the pregnenolone RIA was 16 pg per assay tube. The intraassay and interassay CVs were 2.5% ($n = 4$) and 3.9% ($n = 5$), respectively.

The concentration of estradiol in the medium was determined by RIA as described elsewhere (38). Using antiestradiol serum no. W1, the sensitivity of estradiol RIA was 1 pg per assay tube. The intraassay and interassay CVs were 6.0% ($n = 5$) and 5.9% ($n = 5$), respectively.

The intracellular levels of cAMP were measured in rat GCs using a commercial EIA kit (Assay Designs Inc.).

Statistical Analysis. Data values are given as the mean ± SEM. Differences between progesterone and estradiol were analyzed by one-way analysis of variance (ANOVA) using the SPSS system, version 11.0 (SPSS Inc., Chicago, IL). Comparisons between group means were made using one-way ANOVA and Duncan's multiple-range test (39). For comparison between two groups, Student's *t* tests were used. A difference between two means was considered statistically significant when $P < 0.05$ and highly significant when $P < 0.01$.

Results

Characterization of AHM-EA Extracts and Their Effects on Progesterone and Estradiol Production in Rat GCs. Fractionation of the AHM-EA was performed using silica gel column chromatography with gradient elution and produced 13 subfractions that were assigned letters A to M. Figure 2A shows the effect of different subfractions of AHM-EA on progesterone and estradiol release in rat GCs. Each of the AHM-EA subfractions E, F,

G, and H at 100 μg/ml inhibited progesterone release in rat GCs compared with the subfraction at 0 μg/ml ($n = 6$, $^*P < 0.05$, $F = 52.36$, Duncan's multiple-range test). Each of the AHM-EA subfractions E, F, G, H, and I at 100 μg/ml inhibited estradiol release in rat GCs compared with the subfraction at 0 μg/ml ($n = 6$, $^*P < 0.05$, $F = 46.55$; Duncan's multiple-range test; preliminary data not shown). These results indicate that the AHM-EA-G and AHM-EA-F subfractions had the most potent inhibitory activity against progesterone secretion. The results also indicated the possible presence of more than one antiprogesterone and antiestradiol chemical in the AHM-EA-G and AHM-EA-F subfractions. HPLC analysis was used to further quantify these antiprogesterone and antiestradiol chemicals. The quantification results revealed that AHM-EA-F contained quercetin, naringenin, and gallic acid, and that AHM-EA-G contained naringenin, quercetin, vanillin, syringaldehyde, gallic acid, syringic acid, and *p*-coumaric acid (Fig. 2B). We also studied the effect of different compounds on progesterone release in rat GCs. The administration of different doses of the compounds naringenin, quercetin, vanillin, syringaldehyde, gallic acid, syringic acid, and *p*-coumaric acid (10 and 100 μg/ml), with or without hCG (0.5 IU/ml), followed by a 2-hr incubation, indicated that naringenin at 100 μg/ml elicited the most potent inhibition of progesterone release by rat GCs.

Effect of Adlay Hull Extract on the Concentration of Plasma Progesterone and Estradiol. The effects of AHM-EA-G injection on plasma progesterone levels are shown in Figure 3A. The levels of plasma progesterone were not altered by saline injection. The plasma concentrations of progesterone were significantly reduced from the saline group between 30 and 180 mins after an intravenous injection of AHM-EA-G (2 mg/ml/kg) compared with the saline group ($n = 6$, $^*P < 0.05$; Student's *t* test). A single intravenous injection of 5 IU/ml/kg hCG stimulated a 1.6-fold increase in the concentration of plasma progesterone at 120 and 180 mins compared with basal level (at 0 min; $n = 6$, $+P < 0.05$, $F = 31.25$; Duncan's multiple-range test). The concentration of plasma progesterone returned to basal levels after 3 hrs following the hCG challenge. Two hours after the administration of AHM-EA-G plus hCG there was a significantly reduced level from the hCG-treated group at 120–180 mins ($n = 5$, $^*P < 0.05$ or $^{**}P < 0.01$; Student's *t* test; Fig. 3A).

The effects of AHM-EA-G injection on plasma estradiol levels are shown in Figure 3B. The levels of plasma estradiol were not altered by a saline injection. The plasma concentrations of estradiol were significantly reduced from the saline group at all time points between 30 and 180 mins after an intravenous injection of AHM-EA-G (2 mg/ml/kg; $n = 5$, $^*P < 0.05$; Student's *t* test). A single intravenous injection of 5 IU/ml/kg hCG stimulated a 2.1-fold increase in the concentration of plasma estradiol at 30 mins compared with basal level (at 0 min; $n = 5$, $+P < 0.05$, $F = 28.22$; Duncan's multiple-range test). The concentration of plasma estradiol returned to basal levels after 1 hr following the hCG challenge. Two hours after the administration of AHM-EA-G plus hCG there was a significantly reduced from hCG-treated group at 30–180 min ($n = 5$, $^*P < 0.05$, Student's *t* test; Fig. 3B).

Effect of Adlay Extracts on Progesterone Release by Rat GCs. The administration of different doses of AHM (0.1–100 μg/ml) with or without hCG (0.5 IU/ml) followed by a 2-hr incubation indicated that AHM at a concentration of 100 μg/ml elicited an inhibition of progesterone release by rat GCs ($n = 6$, $^*P < 0.05$ or $^{**}P < 0.01$, $F = 27.68$ and 20.15; Duncan's multiple-range test; Fig. 4A) compared with AHM at 0 μg/ml. Administration of hCG, 8-Br-cAMP (10^{-4} *M*), or forskolin (10^{-5} *M*) alone markedly stimulated progesterone release in GCs ($n = 6$, $++P < 0.01$; Student's *t* test; Fig. 4A) compared with the vehicle group. Furthermore, AHM (100 μg/ml) inhibited not only basal but also 8-Br-cAMP–stimulated (10^{-4} *M*) and forskolin-stimulated (10^{-5} *M*) progesterone release in rat GCs ($n = 6$, $^*P < 0.01$, $F = 27.68$, 18.24, and 20.19; Duncan's multiple-range test). Neither ATM (100 μg/ml) nor PAM (100 μg/ml) had an inhibitory effect on progesterone release in rat GCs (data not shown).

Effect of Subfractions or Compounds from AHM on Progesterone Release by Rat GCs. In order to confirm the inhibitory effect of the major active fraction in adlay hulls on progesterone production, AHM was further partitioned into four fractions: AHM-Wa, AHM-Bu, AHM-EA, and AHM-Hex. (see flow chart in Fig. 2A). After a 2-hr incubation following the administration of different doses of AHM-EA (0.1–100 μg/ml) with or without hCG (0.5 IU/ml), AHM-EA at a concentration of 100 μg/ml elicited an inhibition of progesterone release by GCs ($n = 6$, $^{**}P < 0.01$, $F = 21.59$ and 20.25; Duncan's multiple-range test) compared with AHM at 0 μg/ml. Furthermore, AHM-EA (100 μg/ml) inhibited not only basal but also 8-Br-cAMP–stimulated (10^{-4} *M*) and forskolin-stimulated (10^{-5} *M*; $n = 6$, $^{**}P < 0.01$, $F = 21.59$, 17.35, and 25.39, Duncan's multiple-range test) progesterone release in GCs (Fig. 4B). However, AHM-Wa (100 μg/ml), AHM-Bu (100 μg/ml), and AHM-Hex (100 μg/ml) did not alter progesterone production *in vitro* (data not shown). Subsequently, we fractionated the AHM-EA portion into 13 subfractions (A to M; Fig. 2A) with normal-phase silica gel column chromatography and determined their activity. Subfractions E to G all had inhibitory effects on progesterone secretion (data not shown). Among these, fraction G was found to be the most potent. The administration of different doses of AHM-EA-G (0.1–100 μg/ml), either with or without hCG (0.5 IU/ml), followed by a 2-hr incubation elicited an inhibition of progesterone release by granulosa cells at 10 μg/ml and 100 μg/ml ($n = 6$, $P < 0.05$ or $P < 0.01$, $F = 23.15$ and 16.54; Duncan's multiple-range test) compared with AHM-EA-G at 0 μg/ml. Furthermore, AHM-EA-G (100 μg/ml) inhibited not only basal but also 8-Br-cAMP–stimulated (10^{-4} *M*) and forskolin-stimulated (10^{-5} *M*; $n = 6$, $^*P < 0.01$, $F = 23.15$,

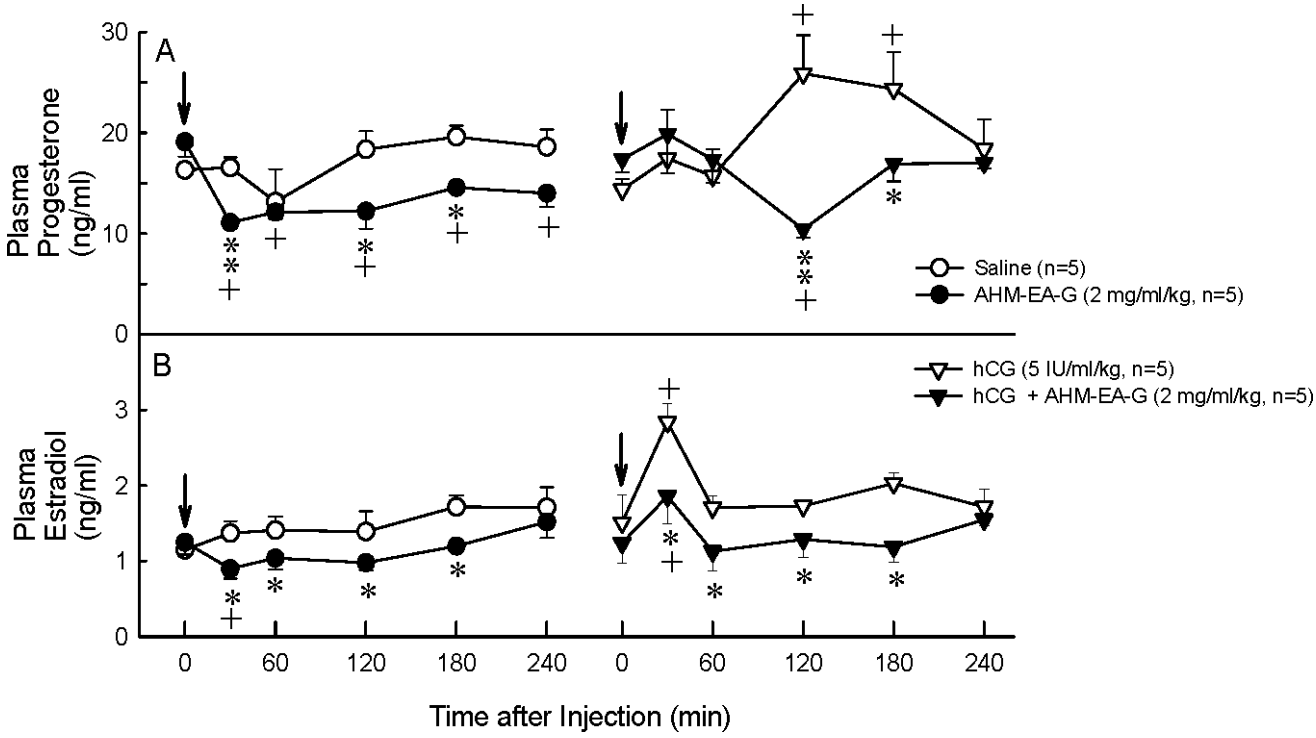

Figure 3. Effects of AHM-EA-G on the basal and hCG-stimulated levels of plasma progesterone (A) and estradiol (B) in female rats. Rats were administered a single intravenous injection of saline, AHM-EA-G (100 μg/ml), hCG (0.5 IU/ml), or hCG (0.5 IU/ml) plus AHM-EA-G (100 μg/ml) *via* a right jugular catheter and were bled at different time intervals after injection. The concentration of progesterone and estradiol was measured by RIA. The arrow (↓) represents a single intravenous injection of saline, AHM-EA-G, hCG, and hCG combined with AHM-EA-G respectively. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with the saline or the hCG-treated group by Student's *t* test. $+P < 0.05$; $++P < 0.01$ compared with time = 0 min by Duncan's multiple-range test. Each value represents the mean ± SEM.

27.32, and 24.47; Duncan's multiple-range test) progesterone release in GCs (Fig. 4C). The administration of different doses of the compounds naringenin, quercetin, vanillin, syringaldehyde, gallic acid, syringic acid, and *p*-coumaric acid (10 and 100 μg/ml), with or without hCG (0.5 IU/ml), followed by a 2-hr incubation indicated that naringenin at 100 μg/ml elicited the most potent inhibition of progesterone release by GCs. The administration of different doses of naringenin (12.5–100 μg/ml), with or without hCG (0.5 IU/ml), 8-Br-cAMP (10^{-4} *M*), or forskolin (10^{-5} *M*), followed by a 2-hr incubation, resulted in an inhibition of progesterone release by GCs in response to naringenin at 25–100 μg/ml ($n = 6$, $^{*}P < 0.05$ or $^{**}P < 0.01$, F = 16.67, 20.52, 17.49, and 22.32; Duncan's multiple-range test; Fig. 5) compared with naringenin at 0 μg/ml.

Effect of AHM-EA-G on Accumulation of cAMP in Rat GCs. With respect to the accumulation of cAMP in response to AHM-EA-G in rat GCs, incubation of the GCs with IBMX (10^{-3} *M*; a phosphodiesterase inhibitor to prevent the inactivation of the intracellular cAMP and to increase accumulation of cAMP in GCs) in the presence or absence of hCG (0.5 IU/ml) or forskolin (10^{-5} *M*) for 1 hr increased cellular cAMP production. The AHM-EA-G (100 μg/ml) inhibited not only IBMX (10^{-3} *M*) but also the IBMX (10^{-3} *M*) + hCG (0.5 IU/ml)–stimulated and IBMX (10^{-3} *M*) + forskolin (10^{-5} *M*)–stimulated cellular cAMP production by rat GCs ($n = 6$, $^{**}P < 0.01$, F = 19.32, 24.74, and 32.87; Duncan's multiple-range test; Fig. 6) compared with AHM-EA-G at 0 μg/ml.

Effect of AHM-EA on the Progesterone Biosynthesis Pathway in Rat GCs. To investigate the effects of AHM-EA on P450scc activity, both 25-OH-cholesterol (10^{-5} *M*) and pregnenolone (10^{-5} *M*) were used to challenge the rat GCs. The 25-OH-cholesterol (10^{-5} *M*) and pregnenolone (10^{-5} *M*) significantly increased progesterone release by the GCs ($n = 6$, $++P < 0.01$; Student's *t* test) compared with the basal group. The AHM-EA (100 μg/ml) and AHM-EA-G (100 μg/ml) inhibited basal, 25-OH-cholesterol–induced (10^{-5} *M*), and pregnenolone-induced (10^{-5} *M*) release of progesterone by the GCs ($n = 6$, $^{**}P < 0.01$, F = 43.68, 31.54, and 35.34; Duncan's multiple-range test; Fig. 7). This result indicates that AHM-EA might have a direct inhibitory effect on P450scc and/or 3β-HSD activity. To further confirm whether AHM-EA affects P450scc and 3β-HSD activities in GCs, trilostane (10^{-5} *M*) was incubated with or without 25-OH-cholesterol (10^{-5} *M*) in order to inhibit the turnover of pregnenolone to progesterone. Also, 25-OH-cholesterol (10^{-5} *M*) with or without trilostane (10^{-5} *M*) significantly increased pregnenolone release by the GCs ($n = 6$, $+P < 0.05$ or $++P < 0.01$; Student's *t* test). However, AHM-EA (100 μg/ml) and AHM-EA-G (100 μg/ml) inhibited basal, 25-OH-cholesterol–induced (10^{-5} *M*),

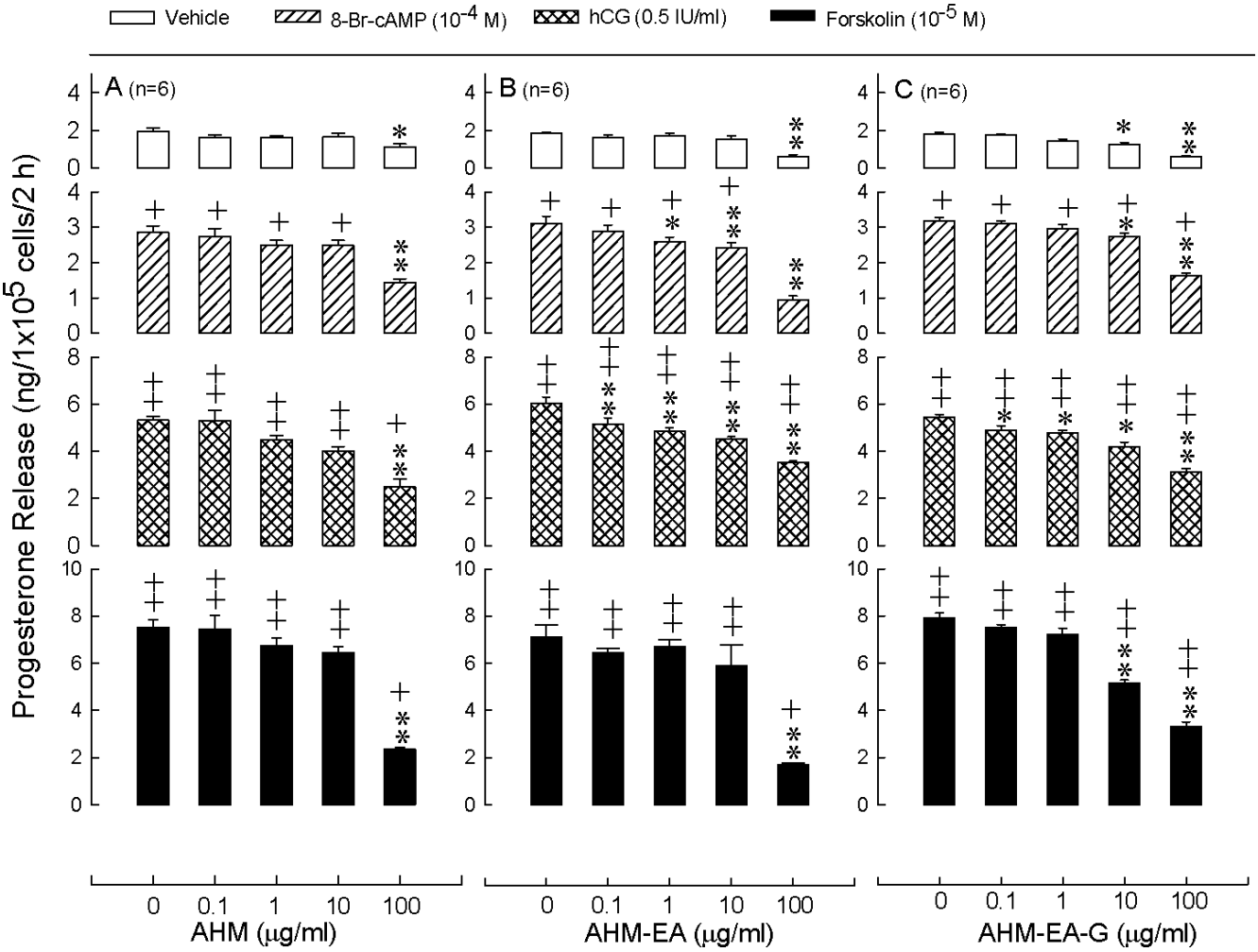

Figure 4. Effects of different doses of AHM (A), AHM-EA (B), and AHM-EA-G (C) on the release of progesterone in the presence or absence of 8-Br-cAMP (10^{-4} *M*)-, hCG (0.5 IU/ml)-, and forskolin (10^{-5} *M*)-stimulated progesterone release in rat GCs. (A–C) Vehicle (open bar), 8-Br-cAMP (hatched bar), hCG (crossed bar), forskolin (closed bar). $^{*}P < 0.05$; $^{**}P < 0.01$ versus AHM = 0 μg/ml by Duncan's multiple-range test. $+P < 0.05$; $++P < 0.01$ compared with the vehicle-treated group by Student's *t* test. Each column represents the mean ± SEM.

and pregnenolone-induced (10^{-5} *M*) release of progesterone by the GCs ($n = 6$, $^{**}P < 0.01$, F = 24.58, 17.88, and 21.54; Duncan's multiple-range test; Fig. 8).

Effect of AHM-EA-G on the Expression of Cytochrome P450scc and StAR mRNA by Rat GCs. In order to investigate whether the inhibitory effects of AHM-EA-G were caused by altered expressions of StAR proteins and P450scc mRNA, after administration of AHM-EA-G (100 μg/ml) and forskolin (10^{-5} *M*) for 30 mins, the mRNA expressions of the StAR proteins and P450scc in rat GCs administrated with AHM-EA-G (100 μg/ml) were investigated. L19 was used as an internal control and was not affected by AHM-EA-G (100 μg/ml). The results demonstrated that StAR protein and P450scc mRNA levels were decreased by a 30-min treatment with 100 μg/ml AHM-EA-G ($n = 3$, $^{*}P < 0.05$ or $^{**}P < 0.01$; Student's *t* test; Fig. 9).

Effect of AHM-EA-G on the Expressions of PKA, Cytochrome P450scc, and StAR Proteins by Rat GCs. To determine the effect of AHM-EA-G on the expressions of PKA, P450scc, and StAR protein in rat GCs, Western blotting was used to determine which steroidogenic enzymes or proteins were altered at the protein level. β-Actin (45 kDa) was used as an internal control and was not affected by AHM-EA-G (100 μg/ml). P450scc (54 kDa), β-actin (45 kDa), PKA (42 kDa), and StAR (30 kDa) bands were detected in rat GCs. The results demonstrated that the levels of each of the PKA, P450scc, and StAR proteins were decreased by a 2-hr treatment with 100 μg/ml AHM-EA-G ($n = 3$, $^{*}P < 0.05$, Student's *t* test; Fig. 10).

Effect of Adlay Extracts and Subfractions on Estradiol Release by Rat GCs. The administration of AHM (1–100 μg/ml) elicited an inhibition of estradiol release by rat GCs ($n = 6$, $^{*}P < 0.05$ or $^{**}P < 0.01$, F = 19.63; Duncan's multiple-range test; Fig. 11). The maximal inhibition caused by 100 μg/ml AHM in rat GCs was 73% (0.07 ± 0.03 ng per 1×10^5 cells per 2 hrs vs. basal release 0.27 ± 0.03 ng per 1×10^5 cells per 2 hrs; $n = 6$, $^{**}P <$

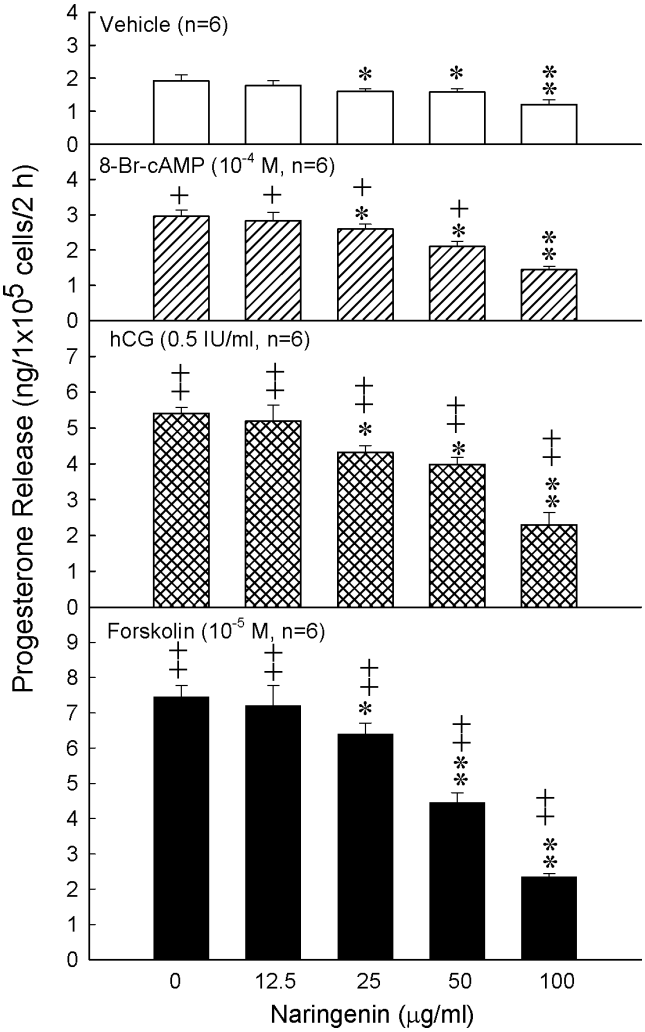

Figure 5. Effects of different doses of naringenin on the release of progesterone in the presence or absence of 8-Br-cAMP–stimulated (10^{-4} *M*), hCG-stimulated (0.5 IU/ml), and forskolin-stimulated (10^{-5} *M*) progesterone release in rat granulosa cells. Vehicle (open bar), 8-Br-cAMP (hatched bar), hCG (crossed bar), and forskolin (closed bar). $^{*}P < 0.05$; $^{**}P < 0.01$ versus AHM = 0 μg/ml by Duncan's multiple-range test. $+P < 0.05$; $++P < 0.01$ compared with vehicle-treated group by Student's *t* test. Each column represents the mean ± SEM.

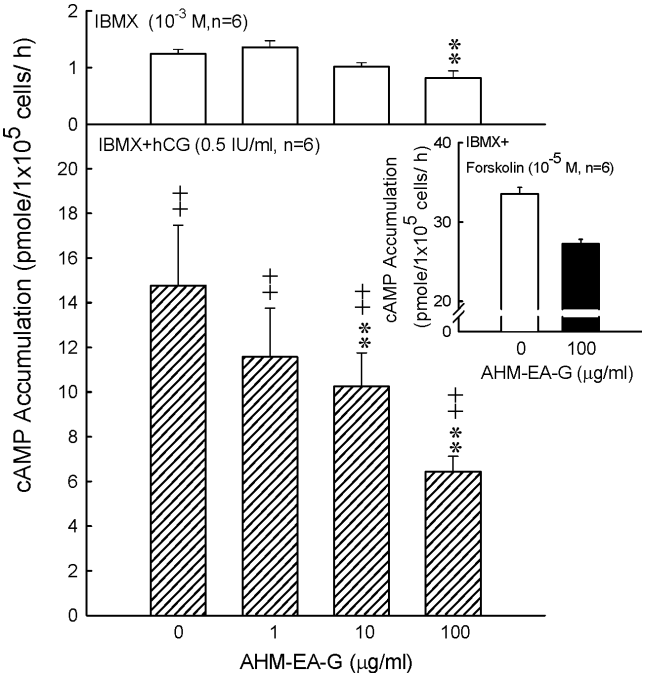

Figure 6. Effects of AHM-EA-G on the IBMX, IBMX + hCG-stimulated, and IBMX + forskolin-stimulated cellular cAMP production by rat GCs. Cells were treated with IBMX (10^{-3} *M*), IBMX (10^{-3} *M*) plus hCG (0.5 IU/ml), or IBMX (10^{-3} *M*) plus forskolin (10^{-5} *M*) in the presence or absence of AHM-EA-G (100 μg/ml), followed by a 1-hr incubation. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with AHM-EA-G at 0 μg/ml by Duncan's multiple-range test. $++P < 0.01$ compared with the vehicle-treated group by Student's *t* test. Each column represents the mean ± SEM.

0.01, F = 19.63, Duncan's multiple-range test; Fig. 11). In order to confirm the inhibitory effect of the major active fraction in adlay hulls on estradiol production, AHM was further partitioned into four fractions: AHM-Wa, AHM-Bu, AHM-EA, and AHM-Hex (see flow chart in Fig. 2A). However, AHM-Wa (100 μg/ml), AHM-Bu (100 μg/ml), and AHM-Hex (100 μg/ml) did not alter estradiol production *in vitro* (data not shown). The administration of AHM-EA (10 and 100 μg/ml) elicited an inhibition of estradiol release by rat GCs ($n = 6$, $^{**}P < 0.01$, F = 19.15; Duncan's multiple-range test; Fig. 11). The maximal inhibition caused by 100 μg/ml AHM in rat GCs was 87% (0.03 ± 0.02 ng per 1×10^5 cells per 2 hrs vs. basal release 0.24 ± 0.02 ng per 1×10^5 cells per 2 hrs; $n = 6$, $^{**}P < 0.01$, F = 19.15; Duncan's multiple-range test; Fig. 11). These results demonstrated that the AHM-EA portion had a significant effect on estradiol secretion in rat GCs. Subsequently, we fractionated the AHM-EA portion into 13 subfractions (A to M; Fig. 2A) using normal-phase silica gel column chromatography and determined their activity. Fractions E to I all had inhibitory effects on estradiol secretion. Among these, fractions F and G were found to be the most active (data not shown). The administration of AHM-EA-F (100 μg/ml) and AHM-EA-G (100 μg/ml) elicited an inhibition of estradiol release by rat GCs ($n = 6$, $^{**}P < 0.01$, F = 24.12 and 22.99; Duncan's multiple-range test; Fig. 11). Neither ATM (100 μg/ml) nor PAM (100 μg/ml) had any inhibitory effect on estradiol release in rat GCs (data not shown).

Effect of Adlay Extracts, Naringenin, and 4-Hydroxyandrostenedione on Aromatase Activity by Rat GCs. An assay was used to determine the effect of AHM-EA-F, AHM-EA-G, and naringenin on aromatase activity in rat GCs. The results demonstrated that the activity of aromatase was decreased by a 2-hr treatment with AHM-EA-G (100 μg/ml), AHM-EA-F (100 μg/ml), AHM-EA-G (100 μg/ml), naringenin (50 and 100 μg/ml), and 4-OHA (50 μ*M*) both in the presence and absence of forskolin (10^{-5} *M*) in rat GCs ($n = 6$, $^{**}P < 0.01$, F = 17.15 and 16.69, Duncan's multiple-range test; Fig. 12).

Hypothetical Scheme of AHM-EA Inhibition of

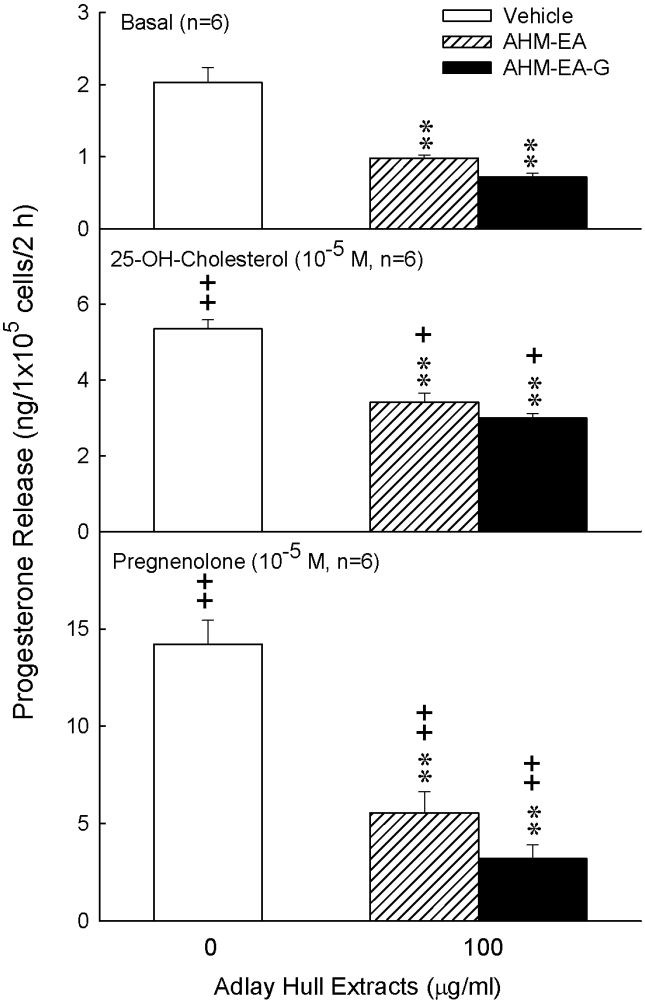

Figure 7. Effects of different doses of AHM-EA on the release of progesterone in the presence or absence of 25-OH-cholesterol–stimulated (10^{-5} *M*) and pregnenolone-stimulated (10^{-5} *M*) progesterone release in rat granulosa cells. $^{*}P < 0.05$; $^{**}P < 0.01$ versus AHM-EA = 0 μg/ml by Duncan's multiple-range test. $+P < 0.05$; $++P < 0.01$ compared with the vehicle-treated group by Student's *t* test. Each column represents the mean ± SEM.

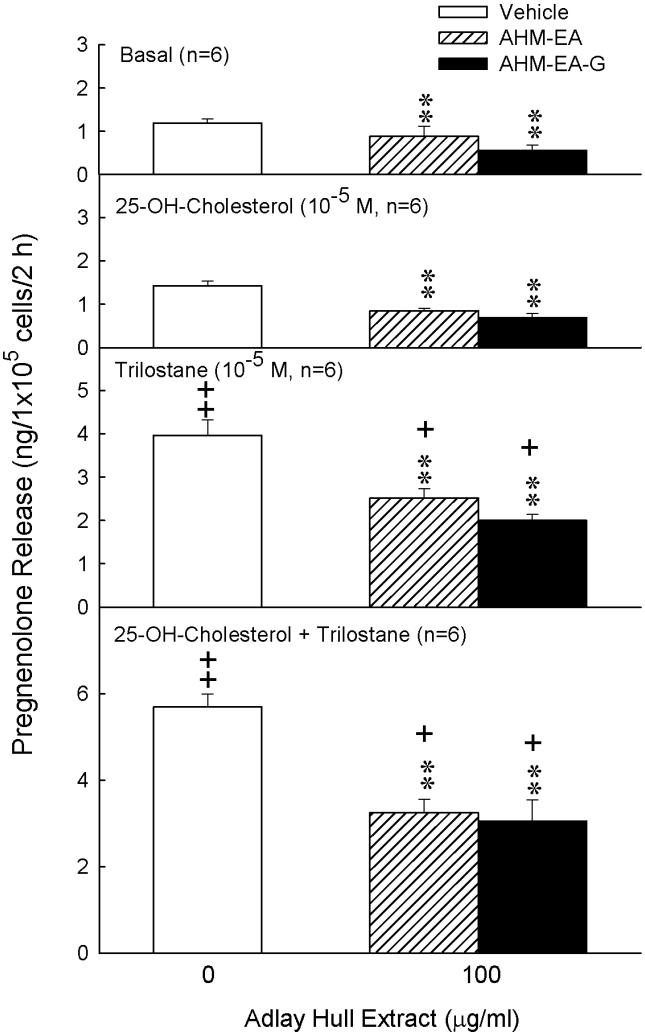

Figure 8. Effects of AHM-EA and AHM-EA-G on progesterone release in rat granulosa cells treated with or without trilostane (10^{-6} *M*), 25-OH-cholesterol (10^{-5} *M*), and trilostane (10^{-6} *M*) plus 25-OH-cholesterol (10^{-5} *M*). $^{*}P < 0.05$; $^{**}P < 0.01$ versus AHM-EA or AHM-EA-G = 0 μg/ml by Duncan's multiple-range test. $+P < 0.05$; $++P < 0.01$ compared with the vehicle-treated group by Student's *t* test. Each column represents the mean ± SEM.

Progesterone or Estradiol Production in Rat Granulosa Cells. The hypothetical scheme (Fig. 13) shows the mechanisms of this effect at the molecular level: AHM-EA inhibition of progesterone or estradiol production in rat GCs. Our results demonstrated that AHM-EA decreased progesterone and estradiol *via* inhibition of (i) the cAMP-PKA signal transduction pathway, (ii) cAMP accumulation, (iii) P450scc and 3β-HSD enzyme activities, (iv) PKA, P450scc and StAR protein expressions and StAR mRNA expression, and (v) aromatase activity in rat GCs.

Discussion

Adlay has long been consumed as both an herbal medicine and as a food supplement in China and Japan. However, the relationship between its action and endocrine functions has been little studied. In the present study, we found that the administration of AHM and its subfractions in rats had the following significant effects: (i) inhibition of the spontaneous and hCG-stimulated secretion of progesterone and estradiol *in vivo* and *in vitro*; (ii) decrease of the secretion of progesterone and estradiol in rat GCs partly *via* a mechanism involving a decrease in the accumulation of cAMP or aromatase activity; (iii) decrease of the activities of P450scc and 3β-HSD; and (iv) decrease of PKA, P450scc, and StAR protein expressions and P450scc and StAR mRNA expressions. The naringenin in the adlay extract was an important component for the decrease in progesterone and estradiol production in rat GCs. To our knowledge, this is the first report demonstrating the effects of adlay hull extracts on steroid hormone secretion *in vivo* and *in vitro*, thus partially explaining the modulatory effects of adlay extracts on female reproductive functions. Conceivably, the different components of adlay seed

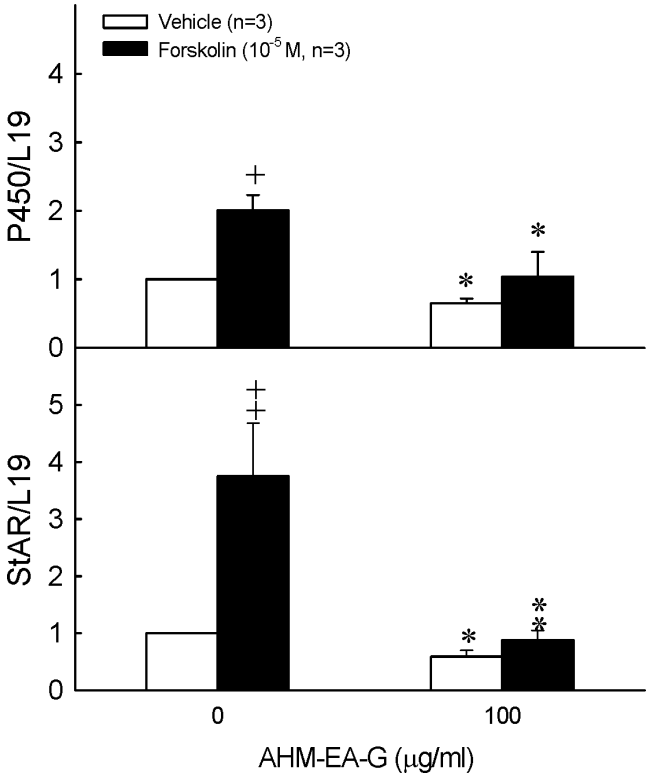

Figure 9. Effect of AHM-EA-G on StAR and P450scc mRNA levels in rat granulosa cells. Cells were treated for 30 mins with AHM-EA-G (100 μg/ml) in the presence or absence of forskolin (10^{-5} *M*), and then mRNA levels of P450scc and StAR were measured by real-time RT-PCR. This experiment was repeated three times with similar results. $^{*}P < 0.05$; $^{**}P < 0.01$ versus AHM-EA-G = 0 μg/ml by Student's *t* test. $+P < 0.05$; $++P < 0.01$ compared with the vehicle-treated group by Student's *t* test. Each column represents the mean ± SEM.

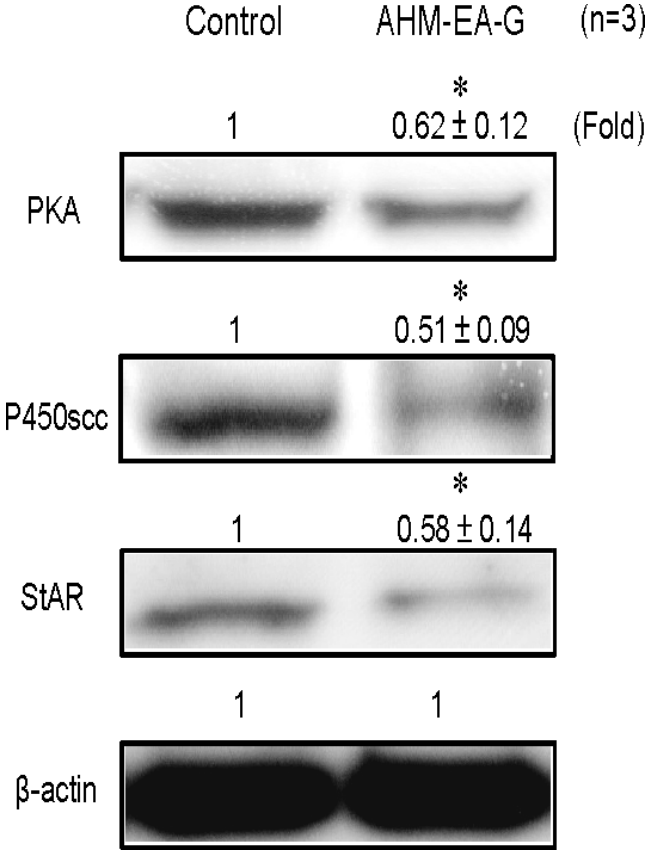

Figure 10. Effect of AHM-EA-G on P450scc, PKA, and StAR protein levels in rat GCs. Cells were treated for 2 hrs with or without AHM-EA-G (100 μg/ml), and then protein levels of P450scc (54 kDa), PKA (42 kDa), and StAR (30 kDa) were measured by Western blotting. The levels of the P450scc, PKA, and StAR protein were inhibited by AHM-EA-G (100 μg/ml). This experiment was repeated three times with similar results. $^{*}P < 0.05$ versus AHM-EA-G = 0 μg/ml by Student's *t* test. Each data point represents the mean ± SEM.

extracts may possess different chemicals that regulate endocrine functions.

It is well established that hCG increases cyclic AMP generation and then stimulates progesterone secretion *in vivo* (40) and *in vitro* (41), and also increases granulosa cellular cAMP content. In the present study, we found that AHM and AHM-EA subfractions inhibited the hCG-stimulated production of progesterone and estradiol by rat GCs both *in vivo* and *in vitro*. The decrease in progesterone was not attributed to the cytotoxicity of the adlay extracts. The administration of AHM, AHM-EA, and AHM-EA subfractions (100 μg/ml) caused no release of lactate dehydrogenase (LDH) from rat GCs (data not shown). We also assessed cell viability using a recovery study. We determined that by removing the inhibitory substance, then refeeding the cells in the presence of a stimulus, we could reverse the inhibition. From our results, we found that AHM, AHM-EA, and AHM-EA subfraction treatments (100 μg/ml) could restore GC progesterone and estradiol production, even if the extracts were removed (data not shown). In order to examine the involvement of the cyclic AMP pathway in the effect of AHM on rat GCs, both an adenylyl cyclase activator, forskolin, and a membrane-permeable cyclic AMP analog, 8-Br-cAMP, were employed. The administration of AHM decreased the 8-Br-cAMP– and forskolin-induced production of progesterone, indicating that the inhibition may be located not only at a post–adenylyl cyclase level but also at a post–cAMP pathway level of progesterone biosynthesis in rat GCs. However, in the present study we measured cAMP directly in rat GCs. We found that AHM-EA-G decreased not only hCG- but also forskolin-induced cellular cAMP production. These results suggested that one of the actions of AHM-EA is beyond the membrane receptor level and involves the inhibition of the formation of cAMP in rat GCs.

In rat GCs, progesterone biosynthesis occurs *via* the conversion of pregnenolone catalyzed by the microsomal enzyme 3β-HSD following the transformation of cholesterol to pregnenolone by P450scc (the rate-limiting enzyme; Ref. 19). In the present study, the administration of either 25-OH-cholesterol or pregnenolone stimulated progesterone secretion in rat GCs. A significant effect of 25-OH-cholesterol (10^{-5} *M*) or pregnenolone (10^{-5} *M*) on progesterone release has previously been observed in GC

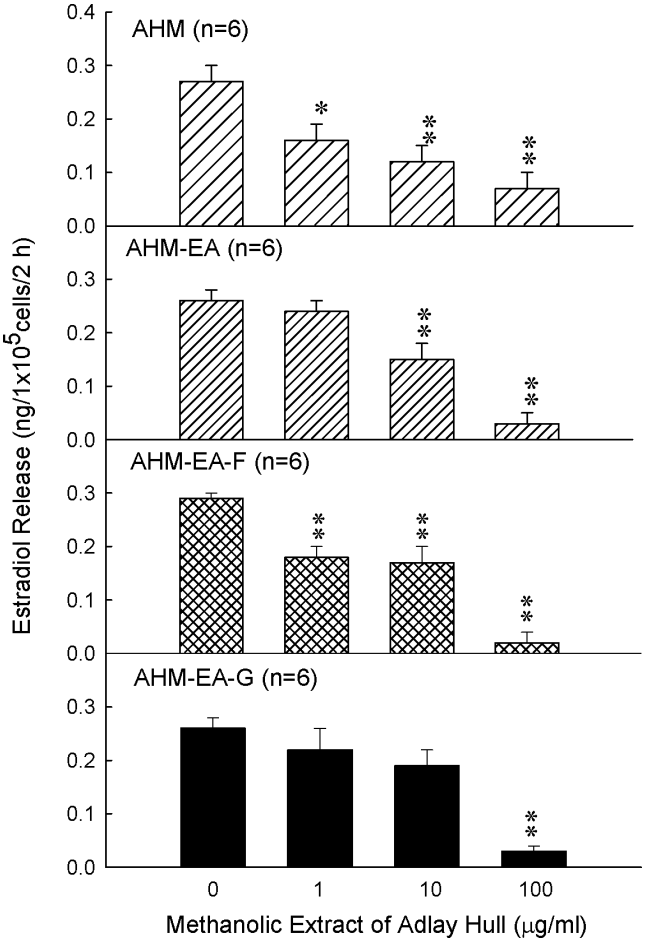

Figure 11. Effects of different doses of AHM, AHM-EA, AHM-EA-F, and AHM-EA-G on the release of estradiol in rat GCs. $^{*}P < 0.05$; $^{**}P < 0.01$ versus AHM, AHM-EA, AHM-EA-F, or AHM-EA-G = 0 μg/ml by Duncan's multiple-range test. Each column represents the mean ± SEM.

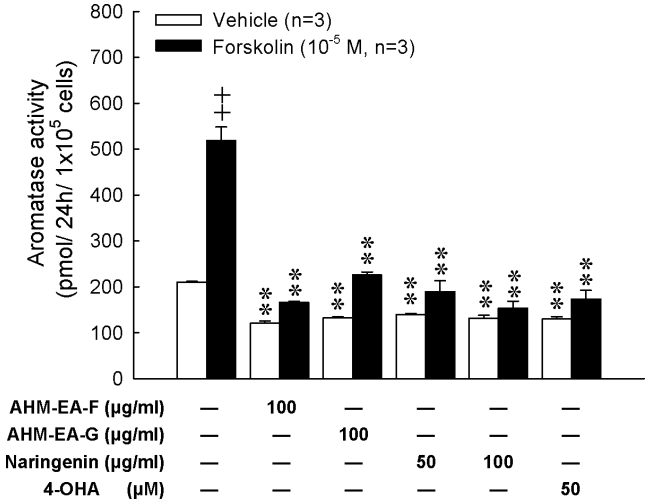

Figure 12. Effects of AHM-EA-F, AHM-EA-G, naringenin, and 4-OHA on aromatase activity in rat GCs. $^{*}P < 0.05$; $^{**}P < 0.01$ versus compound = 0 μg/ml by Duncan's multiple-range test. $^{++}P < 0.01$ compared with the vehicle-treated group by Student's *t* test. Each column represents the mean ± SEM.

culture (22, 29). However, the administration of AHM-EA inhibited the progesterone production caused by 25-OH-cholesterol or pregnenolone. These data suggested that the function of P450scc and/or 3β-HSD might be affected by AHM-EA or AHM-EA-G. To further examine whether the function of either P450scc or 3β-HSD is altered by AHM-EA or AHM-EA-G, we administrated 25-OH-cholesterol with or without AHM-EA or AHM-EA-G. After inhibiting the function of 3β-HSD by trilostane, the pregnenolone accumulation in rat GCs was measured and used as an index of P450scc activity. The present results revealed that both AHM-EA and AHM-EA-G inhibited pregnenolone accumulation in rat GCs. These results suggest that AHM-EA and AHM-EA-G inhibit progesterone secretion in rat GCs, at least in part, by a reduction of P450scc and 3β-HSD activities in progesterone steroidogenesis.

AHM is a crude extract containing many specific chemical components that regulate endocrine functions. In order to confirm which chemical characters decrease progesterone secretion by rat GCs, we partitioned AHM into four fractions. The present results indicated that AHM-EA could decrease both basal and hCG-stimulated progesterone secretion by rat GCs. AHM-EA and AHM-EA-G inhibited the forskolin- and 8-Br-cAMP–induced production of progesterone in rat GCs. AHM-EA-G also inhibited the forskolin- and hCG-induced production of cAMP in rat GCs, which implied that AHM, AHM-EA, or AHM-EA-G possesses some specific chemicals that decrease adenylyl cyclase activity and cAMP function in rat GCs.

The biosynthesis of estradiol involves the initial formation of testosterone from androstenedione, followed by the conversion of testosterone to estradiol *via* aromatase catalyzation (42). We found that AHM, AHM-EA, and the AHM-EA subfractions reduced progesterone and estradiol production. The cause of the reduction of estradiol may be a decreased aromatase activity or substrate supplementation during estrogen synthesis. Our results demonstrated that AHM, AHM-EA, and the AHM-EA subfractions reduced aromatase activity, thereby inhibiting estradiol synthesis.

Progesterone and estradiol are essential for blastocyst implantation and the maintenance of pregnancy in several species. Inhibition of the biosynthesis of both progesterone and estradiol or a blockade of receptor binding will affect endometrial development and function (43–45). Some reports have suggested that adlay seeds should not be consumed during pregnancy, although no supporting scientific evidence was provided (18). It has been reported that AHM possesses some low–molecular weight and moderately polar substances that have antioxidative effects (10). Moreover, it has been demonstrated that there are at least six classes of chemical constituents of AHM: phenolic acid, lignan, flavonoids, polyphenols, polysaccharides, and phytosterols (4, 9, 46). Flavonoid phytochemicals have been demonstrated to exhibit an inhibitory effect on steroidogenic

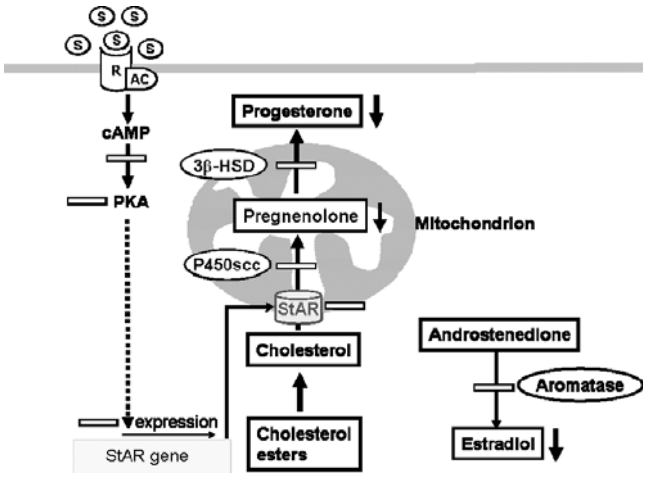

Figure 13. Hypothetical scheme of AHM-EA inhibition of progesterone or estradiol production in rat granulosa cells. In the present study, we found that AHM-EA could block the steroidogenesis pathway in different steps, including: (i) the cAMP-PKA signal transduction pathway, (ii) cAMP accumulation, (iii) P450scc and 3β-HSD enzyme activities, (iv) PKA, P450scc, and StAR protein expressions and StAR mRNA expression, and (v) aromatase activity in rat GCs. S, substances; R, receptor; AC, adenylyl cyclase; PKA, protein kinase A; – indicates that AHM-EA could inhibit this step.

enzymes in human adrenocortical H295 cells (47). We suggest that the flavonoid phytochemicals in AHM and AHM-EA may play an important role in inhibiting progesterone or estradiol secretion in rat GCs. Phenolic compounds, such as *p*-coumaric acid, gallic acid, and ferulic acid, have also been isolated and quantified in AHM (9, 25). *p*-Coumaric acid has been found to have antifertility effects during early gestation in mice (48). It also has been demonstrated to be a reproductive inhibitor in male rats (49). In the present study, AHM, AHM-EA, and AHM-EA subfractions E, F, G, H, and I inhibited progesterone or estradiol secretion in GCs (data not shown). We further quantified the components in the AHM-EA-F and AHM-EA-G subfractions. The AHM-EA-F subfraction contained quercetin, naringenin, and gallic acid, whereas the AHM-EA-G subfraction contained naringenin, quercetin, vanillin, syringaldehyde, gallic acid, syringic acid, and *p*-coumaric acid. In the present study, we demonstrated that naringenin, one of the flavonoid phytochemicals, inhibits progesterone secretion by rat GCs. Naringenin has previously been shown to inhibit HMG-CoA reductase *in vitro* (50, 51) and to downregulate CYP1A1 gene expression in murine Hepa-1c1c7 cells (52). Naringenin also inhibits aromatase activity in human endometrial stromal cells (53). Taken together, the results suggest that AHM contains several different components that inhibit progesterone and estradiol production in rat GCs. These results provided some evidence to explain why adlay should not be consumed during pregnancy.

In summary, the present data demonstrate that the partitioned fractions of adlay extract cause hypogonadism both *in vitro* and *in vivo*. The reduction of the production of progesterone and estradiol is due in part to a decrease of adenylyl cyclase activity, cAMP functions, P450scc and 3β-HSD activity, and protein and mRNA expressions in rat GCs (Fig. 13).

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