Effect of Polyphenols on Production of Steroid Hormones from Human Adrenocortical NCI-H295R Cells

Eri Hasegawa,^a Saori Nakagawa,^a Momoe Sato,^a Eiichi Tachikawa,^b and Susumu Yamato^{*,a}

^aDepartment of Bio-Analytical Chemistry, Niigata University of Pharmacy and Applied Life Sciences; 265–1 Higashijima, Akiha-ku, Niigata 956–8603, Japan; and ^bDepartment of Endocrine and Neural Pharmacology, Tokyo University of Pharmacy and Life Sciences; 1432–1 Horinouchi, Hachioji, Tokyo 192–0392, Japan.

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Modulating steroid hormone levels is a curative and preventive measure for Cushing's syndrome, aldosteronism, and various stress-triggered symptoms. Polyphenols have been reported to inhibit steroidogenic enzymes such as 3 β -hydroxysteroid dehydrogenase (3 β -HSD) and aromatase. However, evidence for their inhibitory effects is fragmentary because it has been determined in studies with small groups of steroid hormones. To investigate the effects of steroids on complete steroidogenic pathways, comprehensive analysis of steroid hormones is necessary. Here we cultured forskolin-stimulated NCI-H295R, a human adrenocortical carcinoma cell line, in the presence of a polyphenol and employed GC-MS to simultaneously determine the levels of nine steroid hormones (pregnenolone, progesterone, deoxycorticosterone, aldosterone, 17 α -hydroxyprogesterone, dehydroepiandrosterone, androstenedione, testosterone, and estradiol) in cell culture supernatant. We found that daidzein, genistein, apigenin, hesperetin, naringenin, and eriodictyol significantly reduced deoxycorticosterone and androstenedione levels ($p < 0.05$), suggesting inhibition of 3 β -HSD by these polyphenols. Apigenin was more potent than other polyphenols in increasing the levels of pregnenolone and 17 α -hydroxyprogesterone, suggesting that it inhibits cytochrome P450 (CYP) 17 and CYP21, as well as 3 β -HSD. Real-time reverse transcription polymerase chain reaction showed that apigenin significantly downregulated the expression levels of 3 β -HSD, CYP17, and CYP21 mRNA ($p < 0.05$). This is the first study to demonstrate the inhibitory effects of apigenin on CYP17 and CYP21.

Key words apigenin; cytochrome P450 (CYP)17; CYP21; pregnenolone; 17 α -hydroxyprogesterone; polyphenol

Steroid hormones are secreted by the adrenal cortex and play pivotal roles in stress response, immune response, anti-inflammatory actions, carbohydrate metabolism, protein catabolism, and electrolyte homeostasis.¹⁾ Disrupting the secretion of these hormones has a serious impact on human health. For example, in Cushing's syndrome, hyperproduction of cortisol results in redistribution of fat to the face (moon face), hypertension, central obesity, and striae distensae. In cases of aldosteronism, hyperproduction of aldosterone induces renal reabsorption of sodium and water, which leads to a rise in circulating blood volume and hypertension. In addition, under stressful conditions, such as those related to deteriorating socioeconomic situations, chronic work-related stress, anxiety, and depression, the neuroendocrine response is upregulated, and the resulting cortisol hyperproduction triggers insulin resistance. Furthermore, the hypothalamic-pituitary-adrenal axis is hyperactivated under chronic stress, which in turn induces abdominal obesity, thereby leading to metabolic syndrome.²⁾

Agents and supplements that modulate steroid hormone levels play pivotal roles in treating the above diseases and conditions. Several food constituents have already been reported as steroid hormone inhibitors. For example, water-soluble low-molecular weight β -1,3-D-glucan was reported to inhibit stress-induced increases in blood corticosterone levels,³⁾ and n-3 fatty acids in fish oil have been shown to suppress stress-induced increases in plasma cortisol levels.⁴⁾ It was also reported that saponin of *Panax ginseng* blocks morphine-induced increases in serum corticosterone levels.⁵⁾ Such food constituents can be consumed easily as part of a daily diet,

are safer than pharmaceutical agents, and have a milder effect.

Polyphenols are common food constituents abundant in tea, legumes, fruits, and vegetables,⁶⁾ and they have been shown to have cardio-protective, anti-cancer, and neuro-protective effects, as well as the ability to reduce blood glucose levels.⁷⁾ Polyphenols can be classed as flavonoids and non-flavonoids; the latter group includes phenolic acids, stilbenes, and lignans. Flavonoids are further divided into flavanols, such as in green tea and chocolate, isoflavones, in legumes such as soy beans, flavanones in citrus fruits, and flavones in celery and parsley. Of them, isoflavones with hydroxyl-groups at C7 and C4', such as daidzein and genistein, are known as phytoestrogens. They show structural similarity to estrogen and exert weak estrogenic actions after binding to the estrogen receptor.^{6,8)} Intake of these phytoestrogens appears to reduce estradiol levels *via* inhibition of aromatase, thereby suppressing breast cancer cells.⁸⁾ Furthermore, genistein is associated with anti-prostate cancer effects, and intake of isoflavones has been reported to reduce prostate volume.⁸⁾ These favorable actions of polyphenols appear to be exerted *via* the inhibition of steroidogenic enzymes. For example, daidzein, genistein, apigenin, and naringenin inhibit 3 β -hydroxysteroid dehydrogenase (3 β -HSD).^{9–11)} while daidzein and genistein inhibit cytochrome P450 (CYP) 21.¹²⁾ In addition, isoflavones inhibit both 3 β -HSD and 17 β -hydroxysteroid dehydrogenase (17 β -HSD).¹³⁾

However, the various actions of polyphenols on steroid hormones have to date being determined through studies with small groups of steroid hormones. This means that findings have been partial and do not demonstrate all possible actions of polyphenols in steroidogenic pathways from cholesterol to steroid hormones. Many enzymes are involved in the

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* To whom correspondence should be addressed. e-mail: yamatos@nupals.ac.jp

steroidogenic pathways, but reports relating to the inhibitory effects of polyphenols on steroidogenesis are limited to the enzymes 3β -HSD and 17β -HSD. On the other hand, few reports address the inhibitory effects of polyphenols on enzymes in the progesterone pathway and the dehydroepiandrosterone (DHEA) pathway. Simultaneous determination of nine steroid hormones—pregnenolone, progesterone, deoxycorticosterone, aldosterone, 17α -hydroxyprogesterone, DHEA, androstenedione, testosterone, and estradiol—will provide comprehensive knowledge on the effect of polyphenols on all steroidogenic pathways.

Several methods have been reported in which steroid hormones were determined by GC-MS.^{14,15)} However, these methods are somewhat complicated because they require two or three reactions, such as *n*-butylboronic cyclization, methyl-oxime derivatization, and trimethylsilyl (TMS) derivatization.

In this study, we aimed to improve the simultaneous determination of nine steroid hormones to make TMS derivatization more simple and accurate, and determined the levels of nine steroid hormones secreted from human adrenocortical NCI-H295R cells stimulated with forskolin in the presence of 11 types of polyphenols. Real-time reverse transcription polymerase chain reaction (RT-PCR) to detect the expression levels of steroidogenic enzyme mRNA was also performed to clarify the inhibition step in the steroidogenic pathways.

MATERIALS AND METHODS

Materials A human adrenocortical carcinoma cell line NCI-H295R was purchased from American Type Culture Collection (Manassas, VA, U.S.A.). Hesperetin (purity, 99%), apigenin (purity, $\geq 97\%$), and forskolin were purchased from Funakoshi (Tokyo, Japan), and (–)-epicatechin (EC; purity, $\geq 98\%$), (–)-epigallocatechin gallate (EGCG; purity, $\geq 98\%$), (–)-epicatechin gallate (ECG; purity, $\geq 98\%$), catechin (purity, 99%), daidzein (purity, $\geq 98\%$), and genistein (purity, 99.9%) were from Cosmo Bio (Tokyo, Japan). Naringenin (purity, $\geq 95\%$) and eriodictyol (purity, $\geq 95\%$) were from Sigma-Aldrich (St. Louis, MO, U.S.A.), and epigallocatechin (EGC; purity, $\geq 98\%$) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). For cell culture, Dulbecco's modified Eagle's medium (DMEM), nutrient mixture F-12 Ham (1:1) medium, and penicillin-streptomycin were purchased from Sigma-Aldrich, ITS+™ Premix and Nu-Serum™ supplements were from BD Biosciences (Bedford, MA, U.S.A.), phosphate buffered saline (PBS) was from Nissui Pharmaceutical (Tokyo Japan), and dimethyl sulfoxide (DMSO) was from Nakalai Tesque (Kyoto, Japan).

DHEA (purity, $>99\%$), estradiol (purity, $>98\%$), androstenedione (purity, $>98\%$), pregnenolone (purity, $>98\%$), progesterone (purity, 99%), deoxycorticosterone (purity, $>98\%$), 17α -hydroxyprogesterone (purity, $\geq 95\%$), and aldosterone (purity, $>95\%$) were purchased from Sigma-Aldrich, and testosterone (purity, $>97\%$) and 5α -cholestane as an internal standard were from Wako Pure Chemical Industries, Ltd. Ethyl acetate (HPLC grade) and *n*-hexane (residual agricultural chemicals and PCB testing grade) were purchased from Wako Pure Chemical Industries, Ltd. Tri-Sil HTP Reagent was from Thermo Fisher Scientific (Waltham, MA, U.S.A.).

For molecular biology, total RNA extraction reagent (RNAiso Plus), PrimeScript RT reagent kit, and SYBR premix

Ex Taq were purchased from Takara Bio (Shiga, Japan). Ethanol (molecular biology grade), isopropyl alcohol (molecular biology grade), and chloroform (JIS high-quality guaranteed grade) were from Wako Pure Chemicals. Diethylpyrocabonate (DEPC)-treated water (genetic engineering grade) was from Nippon Gene (Tokyo, Japan).

GC-MS Analysis GC-MS was performed using the GCMS-QP2010 plus mass spectrometer (electron ionization (EI) method) with the AOC-20s auto-sampler, AOC-20i auto-injector (all from Shimadzu, Kyoto, Japan), and a DB-5MS column (length, 30 m; inner diameter, 0.25 mm; film thickness, 0.25 μ m; Agilent Technologies, Santa Clara, CA, U.S.A.). GC conditions were as follows: carrier gas, helium; flow rate, 0.96 mL/min; injection temperature, 230.0°C; injection mode, splitless; initial column temperature, 180°C; column temperature program, hold at 180°C for 1 min, heat at 20°C/min to 292°C, hold at 292°C for 3 min, heat at 1°C/min to 295°C, heat at 10°C/min to 300°C, and hold at 300°C for 15 min. Selected ion monitoring was performed to measure DHEA at *m/z* 304, 270, 360, estradiol at *m/z* 285, 326, 416, androstenedione at *m/z* 286, 244, testosterone at *m/z* 226, 270, 360, pregnenolone at *m/z* 298, 332, 388, progesterone at *m/z* 272, 229, 314, 17α -hydroxyprogesterone at *m/z* 269, 402, 359, deoxycorticosterone at *m/z* 299, 387, aldosterone at *m/z* 311, 401, 414, and 5α -cholestane at *m/z* 372, 217, 357. The underlined *m/z* value was used for the quantification ion.

Effects of Polyphenols on Levels of Steroid Hormones Produced by Forskolin-Stimulated Cells NCI-H295R cells (2×10^6 cells) were seeded in a 25 cm² flask and cultured for 72 h in DMEM:F-12 containing 1% ITS+™ Premix, 2.5% Nu-Serum™, and 1% penicillin-streptomycin (culture medium). Cells were washed with phosphate buffered saline twice and treated with forskolin (final concentration, 3 μ M) in the presence or absence of 30 μ M of a polyphenol (EGCG, ECG, EGC, EC, catechin, daidzein, genistein, apigenin, hesperetin, naringenin, or eriodictyol; Fig. 1) in the serum-free medium for 24 h to obtain cell culture supernatant samples.¹⁶⁾ For daidzein, genistein, apigenin, hesperetin, naringenin, and eriodictyol, which changed steroid hormone levels, a series of concentrations (0, 1, 3, 10, 30 μ M) were tested to determine whether their effects were dose-dependent. Stock solutions of forskolin and each polyphenol were prepared in DMSO, and the concentrations of DMSO in cell culture were $<0.125\%$.

GC-MS of Steroid Hormones in Cell Culture Supernatant Samples A cell culture supernatant (500 μ L) was placed in a centrifuge tube containing the internal standard (0.25 μ g).¹⁴⁾ After adding 1.5 mL of ethyl acetate, the tube was shaken for 5 min and centrifuged at 3000 rpm for 5 min. The resulting top layer (ethyl acetate) was subjected to solvent extraction twice. After removing the solvent under nitrogen gas flow, 50 μ L of Tri-Sil HTP Reagent was added to perform TMS derivatization for 30 min at 60°C under a nitrogen atmosphere. After removing the solvent under nitrogen gas flow, the remaining residue was dissolved in 50 μ L of *n*-hexane, and 3 μ L of the solution was injected onto the GC-MS column for simultaneous determination of nine types of steroid hormones.

Expression of Steroidogenic Enzyme mRNA by Real-Time RT-PCR Analysis NCI-H295R cells were cultured for 72 h in the culture medium and washed as described above. Cells were then cultured in the serum-free medium containing forskolin (final concentration, 30 μ M) with or without apigenin

Fig. 1. Skeletal Formulae of Polyphenols

(-)-Epigallocatechin gallate (1), (-)-epicatechin gallate (2), (-)-epigallocatechin (3), (-)-epicatechin (4), (+)-catechin (5), daidzein (6), genistein (7), apigenin (8), hesperetin (9), naringenin (10), and eriodictyol (11)

Table 1. Primers and PCR Conditions

Gene		Nucleotide sequence (5'→3')	PCR condition			
			Denaturation	Annealing	Elongation	Cycles
<i>3β-HSD</i>	F	GCGGCTAATGGGTGGAATCTA	94°C, 20 s	54°C, 20 s	72°C, 40 s	50
	R	CATTCTTGTTTCAGGGCCTCAT				
<i>CYP17</i>	F	AGCCGCACACCAACTATCAG	95°C, 15 s	64°C, 60 s	72°C, 30 s	50
	R	TCACCGATGCTGGAGTCAAC				
<i>CYP21</i>	F	ACCTCAGTTTCTCCTTTATTGC	95°C, 20 s	95°C, 30 s	60°C, 30 s	40
	R	AGAGCCAGGGTCCTTCAC				
<i>hGAPDH</i>	F	ATGCCAGTGAGCTTCCCGTCAGC	95°C, 30 s	60°C, 30 s	72°C, 30 s	45
	R	GGTATCGTGGAAGAACTCATGAC				

Key: F, forward; R, reverse.

(final concentration, 1000 μ M) for 24 h. After washing with PBS, cells were dissolved in 1 mL of RNAiso plus and stored at -20°C until use. RNA extraction and reverse transcription were performed according to manufacturer instructions. Briefly, cell samples (in RNAiso plus) were mixed well with

chloroform and centrifuged. RNA was recovered from the top layer by isopropanol precipitation. The RNA pellet was washed with ethanol and dissolved in DEPC-treated water. Reverse transcription was performed on a 96-well plate at 37°C for 15 min: each well contained 5 μ L of an appropriately

Fig. 2. Chromatogram of Standards and Cell Culture Supernatant

(A) Standards, (B) forskolin-stimulated cell culture supernatant. (a) Dehydroepiandrosterone (retention time, 8.5 min), (b) estradiol (9.1 min), (c) androstenedione (9.2 min), (d) testosterone (9.3 min), (e) pregnenolone (9.8 min), (f) 5 α -cholestane (internal standard, 10.7 min), (g) progesterone (10.7 min), (h) 17 α -hydroxyprogesterone (11.5 min), (i) deoxycorticosterone (14.0 min) (j) aldosterone (14.9 min).

Table 2. Calibration and Assay Validation of Nine Steroids Using GC-MS

Steroid	Linearity	Range (ng/mL)	Correlation coefficient (R^2)	Recovery (%)	LOQ (ng/mL)	LOD (ng/mL)
Dehydroepiandrosterone (DHEA)	$y=8.079x+0.033$	1.2–250	0.997	105.0 ± 4.5	1.2	0.4
Estradiol	$y=33.90x+0.063$	0.2–250	0.997	97.2 ± 4.3	0.2	0.05
Androstenedione	$y=4.835x-0.004$	2.5–250	0.999	107.5 ± 6.7	2.5	0.8
Testosterone	$y=8.362x+0.067$	2.5–250	0.993	98.5 ± 5.5	2.5	0.7
Pregnenolone	$y=8.283x+0.050$	1.9–250	0.995	98.3 ± 4.1	1.9	0.6
Progesterone	$y=3.668x-0.004$	18.4–250	0.999	100.7 ± 8.6	18.4	5.5
17 α -Hydroxyprogesterone	$y=1.852x-0.007$	10.2–250	0.999	105.2 ± 13.3	10.2	3.1
Deoxycorticosterone	$y=10.52x-0.080$	3.6–250	0.997	98.8 ± 8.9	3.6	0.2
Aldosterone	$y=8.722x-0.069$	3.0–250	0.998	104.1 ± 11.2	3.0	0.9

The LOD and the LOQ were determined as the concentration of nine steroid hormones giving a signal-to-noise ratio of 3 for LOD and 10 for LOQ. Recovery rate is expressed as mean $\pm$ S.D. ($n=5$).

diluted PrimeScript RT Master Mix solution and 5 μ L of RNA solution. The resulting cDNA solutions were further subjected to real-time PCR using SYBR Premix Ex Taq and a primer pair detecting 3 β -HSD, CYP17, and CYP21^{17–20} (Table 1) on a

96-well plate. Each well contained 1 μ L of the cDNA solution and 24 μ L of the PCR mix. Threshold cycle (C_t) values were determined by the second derivative maximum method.²¹ The housekeeping gene *hGAPDH* was used for normalization of

Fig. 3. Effects of 11 Polyphenols on the Levels of Steroid Hormones Produced by Forskolin-Stimulated NCI-H295R Cells

(A) Pregnenolone, (B) dehydroepiandrosterone (DHEA), (C) deoxycorticosterone, (D) androstenedione, and (E) 17 α -hydroxyprogesterone NCI-H295R cells (2×10^6 cells) were cultured in the presence of 3 μ M forskolin with or without 30 μ M of one of the following polyphenols for 24 h: daidzein (D), genistein (G), apigenin (A), hesperetin (H), naringenin (N), eriodictyol (E), (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), or (-)-epigallocatechin gallate (EGCG). Cell culture supernatant samples were subjected to GC-MS to determine steroid levels. Data are expressed as mean $\pm$ standard error values ($n=3$). Levels of each steroid hormone stimulated with forskolin in the absence of polyphenols were assigned a value of 100%. *Significance $p < 0.05$ by Student's t -test.

real-time RT-PCR.

Data Analysis Data are expressed as mean $\pm$ standard error values and analyzed for statistical significance using the Student's t -test.

RESULTS

Chromatograms of the nine steroid hormones and internal

standard are shown in Fig. 2A. Calibration curves, ranges, the correlation coefficient, limit of detection (LOD), limit of quantitation (LOQ), and the recovery rates from the cell culture supernatant ($n=5$) are shown in Table 2. The LOD and LOQ were determined as the concentration of the nine steroid hormones giving a signal-to-noise ratio of 3 for LOD and 10 for LOQ. The recovery rates from the cell culture supernatant were estimated for a spiked concentration of 250 ng/mL. A

Fig. 4. Dose-Dependent Effects of Polyphenols on the Levels of Steroid Hormones Produced by Forskolin-Stimulated NCI-H295R Cells

(A) Pregnenolone, (B) dehydroepiandrosterone (DHEA), (C) deoxycorticosterone, (D) androstenedione, and (E) 17α -hydroxyprogesterone NCI-H295R cells (2×10^6 cells) were cultured in the presence of $3 \mu\text{M}$ of forskolin with or without 0 – $30 \mu\text{M}$ of one of the following polyphenols for 24 h: daidzein (closed circle), genistein (open circle), apigenin (closed square), hesperetin (open square), or eriodictyol (open triangle). Cell culture supernatant samples were subjected to GC-MS to determine the levels of steroid hormones. Data are expressed as mean $\pm$ standard error values ($n=3$). Levels of each steroid hormone stimulated with forskolin in the absence of polyphenols were assigned a value of 100%. *Significance at $p < 0.05$ by Student's t -test.

chromatogram of forskolin-stimulated cell culture supernatant is shown in Fig. 2B. The chromatogram clearly shows the separation of nine steroid hormones and no influence of the matrix. Calibration curves using the internal standard method showed excellent linearity for all nine steroid hormones (correlation coefficient, 0.993 – 0.999). The recovery rates from the cell culture supernatant were also good, ranging from $97.2 \pm$

4.3 to $107.5 \pm 6.7\%$.

Pregnenolone, DHEA, deoxycorticosterone, androstenedione, and 17α -hydroxyprogesterone levels were significantly increased by 3.61 -, 4.33 -, 3.84 -, 1.66 -, and 1.89 -fold, respectively, by the stimulation of NCI-H295R cells with $3 \mu\text{M}$ of forskolin ($p < 0.05$; Fig. 3). Estradiol, testosterone, progesterone, and aldosterone levels were below the limit of detection

Fig. 5. Effects of Apigenin on the Expression Levels of Steroidogenic Enzyme mRNA

(A) 3β-Hydroxysteroid dehydrogenase (3β-HSD), (B) CYP17, and (C) CYP21 NCI-H295R cells (2×10^6 cells) were cultured in the presence of $30 \mu\text{M}$ of forskolin and $1000 \mu\text{M}$ of apigenin for 24h. The mRNA expression levels of three steroidogenic enzymes were determined by real-time reverse transcription polymerase chain reaction. Data are expressed as mean $\pm$ standard error values ($n=3$). The relative rate of mRNA expression in the absence of both forskolin-stimulation and apigenin is expressed as 1. *Significance at $p < 0.05$ by Student's *t*-test.

of the corresponding calibration curves. We next examined the effects of the 11 polyphenols at the final concentration of $30 \mu\text{M}$ on the steroid hormone levels produced by the forskolin-stimulated cells. Pregnenolone levels were significantly higher in the presence of daidzein, genistein, apigenin, naringenin, and eriodictyol than in the absence of polyphenols ($p < 0.05$; Fig. 3A). Similarly, the levels of DHEA were significantly higher in the presence of daidzein, genistein, apigenin, hesperetin, and naringenin than in the absence of polyphenols ($p < 0.05$; Fig. 3B). On the other hand, the levels of deoxycorticosterone were significantly lower in the presence of daidzein, genistein, apigenin, hesperetin, naringenin, and eriodictyol than in the absence of polyphenols ($p < 0.05$; Fig. 3C). Androstenedione levels were significantly lower in the presence of daidzein, genistein, apigenin, hesperetin, naringenin, and eriodictyol than in the absence of polyphenols ($p < 0.05$; Fig. 3D). 17α -Hydroxyprogesterone levels were significantly lower in the presence of daidzein, but significantly higher in the presence of genistein, apigenin, hesperetin, naringenin, and

eriodictyol than in the absence of polyphenols ($p < 0.05$; Fig. 3E). The effects of catechins, EC, EGC, ECG, and EGCG on steroid hormone levels produced by forskolin-stimulated NCI-H295R cells were negligible (Fig. 3).

In cultured forskolin-stimulated NCI-H295R cells, genistein, apigenin, and eriodictyol significantly increased the levels of pregnenolone in the cell culture supernatant in a dose-dependent manner ($p < 0.05$; Fig. 4A). Similarly, daidzein, genistein, and apigenin significantly increased the levels of DHEA in a dose-dependent manner ($p < 0.05$; Fig. 4B). On the other hand, daidzein, genistein, apigenin, hesperetin and naringenin significantly decreased the levels of deoxycorticosterone in a dose-dependent manner ($p < 0.05$; Fig. 4C). Daidzein, genistein, and apigenin significantly decreased the levels of androstenedione in a dose-dependent manner ($p < 0.05$; Fig. 4D). Daidzein significantly decreased ($p < 0.05$), while genistein, apigenin, hesperetin, and naringenin significantly increased ($p < 0.05$) the levels of 17α -hydroxyprogesterone in a dose-dependent manner (Fig. 4E).

Fig. 6. Apigenin's Points of Intervention in Steroidogenesis

In summary, daidzein dose-dependently increased the DHEA levels, while dose-dependently decreasing the levels of androstenedione, deoxycorticosterone, and 17 α -hydroxyprogesterone, suggesting inhibition of 3 β -HSD and CYP21 by this polyphenol. Genistein increased the levels of pregnenolone, DHEA, and 17 α -hydroxyprogesterone, while dose-dependently decreasing the levels of androstenedione and deoxycorticosterone, suggesting inhibition of 3 β -HSD, CYP17, and CYP21. Hesperetin decreased the levels of androstenedione and deoxycorticosterone, while slightly increasing 17 α -hydroxyprogesterone levels, suggesting inhibition of 3 β -HSD, CYP17, and CYP21. Naringenin dose-dependently decreased deoxycorticosterone levels, while dose-dependently increasing 17 α -hydroxyprogesterone levels, suggesting inhibition of 3 β -HSD, CYP17, and CYP21. Eriodictyol slightly, but dose-dependently increased pregnenolone levels, while slightly decreasing androstenedione and deoxycorticosterone levels, suggesting inhibition of 3 β -HSD, CYP17, and CYP21. Apigenin markedly increased pregnenolone and 17 α -hydroxyprogesterone levels and slightly increased DHEA levels, while dose-dependently decreasing androstenedione

and deoxycorticosterone levels, suggesting inhibition of 3β -HSD, CYP17, and CYP21. There are reports of daidzein, genistein, apigenin, and naringenin inhibiting 3β -HSD,⁹⁻¹¹ and similar reports of daidzein and genistein inhibiting CYP21.¹² However, the inhibitory effect of apigenin on CYP17 and CYP21 has not yet been reported. The present study revealed a new inhibitory effect of apigenin on CYP17 and CYP21. Consequently, we examined the inhibitory effects of apigenin on CYP17 and CYP21 mRNA by RT-PCR.

The expression of 3β -HSD, *CYP17*, and *CYP21* mRNA upon forskolin stimulation with or without apigenin was significantly higher in forskolin-stimulated cells than in the controls ($p<0.05$). It has been reported that daidzein inhibits 3β -HSD and CYP21, but not CYP17.¹²⁾ Therefore, daidzein was used as a positive control for the inhibitory substance. Daidzein significantly decreased forskolin-induced increases in the expression levels of 3β -HSD and *CYP21* mRNA ($p<0.05$), but did not decrease forskolin-induced increases in the expression levels of *CYP17* (Fig. 5). Next, apigenin significantly decreased forskolin-induced increases in the expression levels of 3β -HSD, *CYP17*, and *CYP21* mRNA ($p<0.05$; Fig. 5) demonstrating the

inhibitory effects of apigenin on the expression of 3β -HSD, CYP17, and CYP21.

DISCUSSION

Several methods have been reported for determining steroid hormones by GC-MS.^{14,15} However, these methods are somewhat complicated because they require two or three reactions, such as *n*-butylboronic cyclization, methyloxime derivatization, and TMS derivatization. In this study, we improved the simultaneously determination of nine steroid hormones by simplifying the process and ensuring accuracy by TMS derivatization using a mixture of hexamethyldisilazane–trimethylchlorosilane–pyridine in a ratio of 2:1:10.

Polyphenols are abundant in the human diet and are of particular interest because they are implicated in protective actions against diseases such as cancer, cerebrovascular diseases, and neurodegenerative diseases.⁷ Several polyphenols have been shown to have inhibitory effects against steroidogenic enzymes; for example, daidzein, genistein, apigenin, and naringenin act against 3β -HSD, while isoflavones act against 3β -HSD and aromatase.^{9–13} In these reports, only one or two steroid hormones have been determined to confirm the inhibitory effects of polyphenols on 3β -HSD and aromatase. Accordingly, we tested the effects of polyphenols on steroidogenic pathways by simultaneously measuring levels of nine steroid hormones. We found that isoflavones (daidzein and genistein), one flavone (apigenin), and three flavanones (hesperetin, naringenin, and eriodictyol) dose-dependently decreased the levels of androstenedione and deoxycorticosterone produced by forskolin-stimulated NCI-H295R cells, suggesting inhibition of 3β -HSD. These inhibitory effects were in good agreement with the results of previous studies.^{9–11} Daidzein carries a hydroxyl group at C7 of the A-ring and competes with 3β -HSD substrates because of its steric structure and strong electron affinity with the active center of 3β -HSD.⁹ Genistein, apigenin, hesperetin, naringenin, and eriodictyol also carry a hydroxyl group at C7 of the A-ring, suggesting that their inhibitory mechanisms are similar to those of daidzein.

The effect of apigenin was particularly prominent on levels of pregnenolone produced by forskolin-stimulated NCI-H295R cells. In addition to the hydroxyl group at C7,⁹ a phenolic B ring at C3 of the pyran ring is important for the inhibitory effects of the isoflavones daidzein and genistein on 3β -HSD.¹³ Flavones such as apigenin do not carry such a phenolic B ring, and this may explain their stronger inhibitory effect. On the other hand, apigenin increased the levels of pregnenolone and 17α -hydroxyprogesterone more than isoflavones, suggesting that apigenin inhibits CYP17, CYP21, and 3β -HSD. Simultaneous determination of steroid hormones has revealed novel inhibitory effects of apigenin on CYP17 and CYP21. Apigenin appeared to inhibit expression of the above three enzymes, as confirmed by quantitative real-time RT-PCR. The inhibitory effects on multiple steroidogenic enzymes were shown by our approach; namely, simultaneously examining the levels of multiple steroid hormones. Indeed, this study is the first to show the inhibitory effects of apigenin on CYP17 and CYP21. Apigenin's points of intervention in steroidogenesis are shown in Fig. 6.

Comprehensive understanding of steroidogenic pathways is important to understand the actions of pharmaceutical agents

and food constituents such as polyphenols on steroid hormone levels. Furthermore, such understanding will contribute to the control of steroid hormone levels in patients with excessive steroid hormone production.

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