

Inhibitory effects of flavonoids on the reduction of progesterone
to 20 α -hydroxyprogesterone in rat liver

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Abstract

The first aim of this study is to characterize the reduction of progesterone in rat liver. Progesterone was mainly reduced to 20 α -hydroxyprogesterone in the cytosolic fraction of rat liver. The amount of 20 α -hydroxyprogesterone formed from progesterone in the cytosolic fraction was significantly larger in the males than in the females and this enzyme reaction proceeded not only in the presence of NADPH, but also in the presence of NADH. Furthermore, we attempted to evaluate the inhibitory effects of 15 flavonoids on the NADPH-dependent reduction of progesterone to 20 α -hydroxyprogesterone in liver cytosol of male rats. The order of the inhibitory potencies was luteolin > apigenin > quercetin > myricetin = fisetin = kaempferol. Other flavonoids exhibited lower inhibitory potencies. Energy-minimized molecular models demonstrated that a planar benzopyrone ring (A and C rings) with a coplanar phenyl ring (B ring) is a structural characteristic determining the inhibitory effects of flavonoids other than isoflavones.

Keywords: Progesterone; Flavonoids; Inhibitory potency; Energy-minimized structure; Rat liver

1. Introduction

Progesterone is essential to establish and sustain pregnancy in mammals. In addition, progesterone is a precursor of biologically active steroids such as androgens, estrogens and corticoids. Thus, the conversion of progesterone into its biologically inactive form, 20 α -hydroxyprogesterone, is probably involved in the maintenance of pregnancy and the regulation of steroid biosynthesis [1–3]. 20 α -Hydroxysteroid dehydrogenase (20 α -HSD) [EC 1.1.1.149] is an NADPH-dependent enzyme that catalyzes the reduction of progesterone to 20 α -hydroxyprogesterone [4,5]. In humans, although the expression of 20 α -HSD mRNA is detected in the ovary, the liver is the major tissue in which the function of 20 α -HSD in the reproductive system remains unclear [6,7]. In rats, 20 α -HSD mRNA is highly expressed in the ovary, whereas it is almost undetectable in the kidney, lung and heart [8].

Flavonoids are natural components that are widely distributed in vegetables, fruits, and green tea. These compounds have been shown to have extensive biological effects such as enzyme inhibition and antioxidant activity. Because flavonoids are structurally similar to steroid hormones, they can inhibit HSDs responsible for biosynthesis or degradation of steroid hormones such as progesterone. For example, of various flavonoids, apigenin is the most potent inhibitor of human placental 17 β -HSD [9]. Furthermore, flavonoids are known to inhibit effectively 17 β -HSD type 5 [10], 3 β -HSD type II [11] and 11 β -HSD [12].

The first aim of the present study is to characterize the reduction of progesterone to 20 α -hydroxyprogesterone in rat liver. Under the obtained experimental conditions, we attempted to evaluate the inhibitory effects of flavonoids listed in Fig. 1 on the reduction of progesterone to 20 α -hydroxyprogesterone in rat liver.

2. Materials and methods

2.1. Chemicals

Progesterone (4-pregnene-3,20-dione), 20 α -hydroxyprogesterone (4-pregnen-20 α -ol-3-one) and 20 β -hydroxyprogesterone (4-pregnen-20 β -ol-3-one) were purchased from Sigma (St. Louis, MO). Flavonoids were obtained from the following sources: morin, myricetin, genistein, taxifolin (racemate), kaempferol, daidzein (Sigma); naringenin and apigenin (Aldrich, Milwaukee, WI); quercetin and luteolin (Wako Pure Chemicals, Tokyo, Japan); fisetin and quercitrin (Tokyo Kasei, Tokyo, Japan). Genistin, (+)-catechin and rutin were donated by Dr. J. Kinjo (Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka, Japan). A planar catechin analogue was synthesized by reacting (+)-catechin with acetone as reported previously [13]. NADPH, NADP, NADH, NAD, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast (Tokyo, Japan). All other chemicals were of reagent grade.

2.2. Animals

Male and female Fischer 344 (Fischer) rats at 8 weeks of age were purchased from Japan SLC (Shizuoka, Japan). The animals had free access to a diet of standard laboratory chow and water. All animal experiments were undertaken in compliance with the guideline principles and procedures of Kumamoto University for the care and use of laboratory animals.

2.3. Preparation of subcellular fractions

Animals were lightly anesthetized and killed by decapitation. The liver quickly excised, perfused with ice-cold 1.15% KCl and homogenized in a Potter-Elvehjem homogenizer with three volumes of 10 mM sodium-potassium phosphate buffer containing 1.15% KCl (pH 7.4). All subsequent procedures were performed at 3–5 °C. The homogenates were centrifuged at 10,000g for 20 min and the resulting supernatants were centrifuged at 105,000g for 60 min to obtain the microsomal pellets and cytosolic fraction. The microsomal pellets were suspended in 10 mM sodium-potassium phosphate buffer containing 1.15% KCl (pH 7.4) and were recentrifuged at 105,000g for 60 min. The microsomal pellets and cytosolic fraction obtained were used as enzyme preparations.

2.4. Enzyme reaction of progesterone

The enzyme reaction of progesterone in subcellular fractions was conducted in an NADPH-generating system consisting of progesterone (0.1 mM), NADP (0.25 mM), glucose-6-phosphate (6.25 mM), glucose-6-phosphate dehydrogenase (0.25 units), MgCl₂ (6.25 mM), enzyme preparations and 100 mM sodium-potassium phosphate buffer (pH 7.4) in a final volume of 2.0 ml. In studying cofactor requirement, NADPH, NADP, NADH or NAD at a concentration of 0.5 mM was added to the mixture except glucose-6-phosphate (6.25 mM), glucose-6-phosphate dehydrogenase (0.25 units), MgCl₂ (6.25 mM). The mixture was incubated at 37 °C for 30 min under aerobic condition, and the reaction was stopped by adding 0.5 ml of 1.0 N HCl to the mixture. The reduction products (20 α -hydroxyprogesterone and 20 β -hydroxyprogesterone) of progesterone were determined by HPLC according to a slightly modified of Swinney et al [14]. HPLC was carried out using a Tosoh DP-8020 HPLC apparatus (Tosoh, Tokyo, Japan) equipped with a Tosoh ODS-80Ts column and a Tosoh UV-8020 monitor (240 nm). Mixture of water-acetonitrile-methanol-tetrahydrofuran (44:28:17:11, v/v) was used

as a mobile phase at a flow rate of 0.6 ml/min. The amount of 20 α -hydroxyprogesterone or 20 β -hydroxyprogesterone formed from progesterone was expressed as pmol/min/mg protein. Protein concentration was estimated by the method of Lowry et al. [15] with bovine serum albumin as the standard.

2.5. Inhibition experiments

Flavonoids were dissolved in methanol and then added to the reaction mixture described above. The final concentration of methanol did not exceed 2 % (v/v), and this concentration did not affect the enzyme reaction. The IC₅₀ value (the concentration of flavonoids required to inhibit the enzyme reaction by 50 %) was determined from linear regression of at least four points in different concentrations.

2.6. Molecular conformation of flavonoids

The energy-minimized structure of flavonoids was calculated by means of molecular mechanics using MM2.

2.7. Statistical analysis

Statistical analysis of data was performed using Student's *t*-test, and *P* < 0.05 was considered to be significant.

3. Results

3.1. Stereoselective reduction of progesterone in liver cytosol and microsomes of male rats

The stereoselective reduction of progesterone in two subcellular fractions (cytosol and microsomes) from the liver of male rats was investigated using the HPLC method. As shown in Fig. 2A, the peak of 20 α -hydroxyprogesterone formed in the cytosolic fraction was much higher than that of 20 β -hydroxyprogesterone, indicating that cytosolic enzyme(s) can mainly catalyze the reduction of progesterone to 20 α -hydroxyprogesterone in rat liver. On the other hand, progesterone was mostly reduced to 20 β -hydroxyprogesterone by microsomal enzyme(s) (Fig. 2B).

3.2. Sex difference and cofactor requirement in the reduction of progesterone to 20 α -hydroxyprogesterone

The reduction of progesterone to 20 α -hydroxyprogesterone in liver cytosol was compared between male and female rats. As shown in Fig. 3, the amount of 20 α -hydroxyprogesterone formed from progesterone was significantly larger in the males than in the females. Furthermore, the cofactor requirement in the reduction of progesterone to 20 α -hydroxyprogesterone was examined in liver cytosol of male rats. In this enzyme reaction, both NADPH and NADH were effective as cofactor: 20 α -hydroxyprogesterone formed was respective 45.5 ± 10.6 and 60.0 ± 5.0 pmol/min/mg protein ($n = 3$).

3.3. Inhibition of progesterone reduction by flavonoids

We investigated the inhibitory effects of 15 flavonoids on the NADPH-dependent reduction of progesterone to 20 α -hydroxyprogesterone in liver cytosol of male rats. Figure 4 shows the inhibition percentages of 15 flavonoids at a concentration of 30 μ M. The IC₅₀ values are summarized in Table 1. The order of the inhibitory potencies was luteolin > apigenin > quercetin > myricetin = fisetin = kaempferol. Other flavonoids exhibited lower inhibitory potencies. Flavones and flavonols were more potent inhibitors than flavanone (naringenin) and flavanonol (taxifolin), although the glycosylated

flavonols such as quercitrin and rutin were poor inhibitors. These results clearly indicate that the C2-C3 double bond in the C ring contributes to the inhibitory potencies of flavones and flavonols. Among the flavonols, quercetin and morin have identical numbers of hydroxyl group in the same positions, except for the substitution of a hydroxyl group for the 2'- and 3'-position in the B (phenyl) ring. However, the inhibitory potency of morin ($IC_{50} > 50 \mu M$) was lower than that of quercetin ($IC_{50} = 21.0 \pm 6.2 \mu M$). Isoflavones and flavan-3-ol [(+)-catechin] were poor inhibitors. The relative lipophilicities of kaempferol, quercetin and myricetin have been reported to decrease with increasing numbers of hydroxyl group in the phenyl ring (see Table 1) [16]. However, the relative lipophilicities of these flavonols appeared to be independent of their inhibitory potencies.

3.4. Molecular conformations of flavonoids

To further elucidate the structure-activity relation for inhibitory potencies of flavonoids, their energy-minimized structures were calculated by means of molecular mechanics using MM2. Figure 5 shows the molecular conformations of apigenin (A) ($IC_{50} = 8.8 \pm 0.8 \mu M$) and naringenin (B) ($IC_{50} > 50 \mu M$), which are the same in the numbers and positions of hydroxyl group. As evident from the two molecular conformations, the pyran ring (C ring) of naringenin, unlike the pyrone ring (C ring) of apigenin, was puckered due to the saturation of the C2-C3 double bond. The molecular conformations of quercetin ($IC_{50} = 21.0 \pm 6.2 \mu M$) and morin ($IC_{50} > 50 \mu M$) were also compared with each other. As shown in Fig. 5, morin (D) had a twisted conformation between its pyrone and phenyl rings compared to quercetin (C). The dihedral angles (C3-C2-C1'-C6') between the pyrone and phenyl rings of morin and quercetin were 48.6° and 36.6° , respectively. Interestingly, the dihedral angles of apigenin and luteolin were 19.9° and 23.0° , respectively. As expected from these dihedral angles, the inhibitory potencies of

apigenin and luteolin were higher than that of quercetin.

3.5. Inhibition of progesterone reduction to 20 α -hydroxyprogesterone by a planar catechin analogue

A planar catechin analogue (B in Fig. 6) has been recently synthesized from (+)-catechin (A in Fig. 6) [13]. Thus, the inhibition of progesterone reduction to 20 α -hydroxyprogesterone by the planar catechin analogue at a concentration of 30 μ M was examined in liver cytosol of male rats. However, the inhibition percentage of the catechin analogue (3.7 ± 4.2 %) was similar to that of (+)-catechin (3.6 ± 4.2 %).

4. Discussion

In the present study, progesterone was confirmed to be mainly reduced to 20 α -hydroxyprogesterone in the cytosolic fraction of rat liver. On the other hand, progesterone was mostly reduced to 20 β -hydroxyprogesterone in the microsomal fraction of rat liver. It should be noted that the reduction products of progesterone generated in the two subcellular fractions exhibit different stereoselectivities. Furthermore, the amount of 20 α -hydroxyprogesterone formed from progesterone in the cytosolic fraction was significantly larger in the males than in the females and this enzyme reaction proceeded not only in the presence of NADPH, but also in the presence of NADH. Rat ovarian 20 α -HSD responsible for the reduction of progesterone to 20 α -hydroxyprogesterone has been reported to have no ability to use NADH as cofactor [17]. Thus, it is possible that rat hepatic 20 α -HSD, unlike rat ovarian 20 α -HSD, requires both NADPH and NADH as cofactor, even though it may consist of two types of NADPH- and NADH-dependent enzymes in the cytosolic fraction of rat liver. Further studies are in progress to

elucidate catalytic properties of 20 α -HSD purified from liver cytosol of male rats.

The present study has revealed structural characteristics of flavonoids necessary for inhibiting the NADPH-dependent reduction of progesterone to 20 α -hydroxyprogesterone in liver cytosol of male rats. On the basis of energy-minimized molecular models, we conclude that a planar benzopyrone ring (A and C rings) with a coplanar phenyl ring (B ring) is a structural characteristic determining the inhibitory effects of flavonoids other than isoflavones. In liver cytosol of female rats, however, the inhibition of progesterone reduction to 20 α -hydroxyprogesterone by flavonoids remains to be examined, although the inhibitory mechanism of flavonoids may be similar to that described in liver cytosol of male rats.

Isoflavones such as daidzein and genistein are structurally analogous to steroid hormones and known to exhibit estrogenic activity [18, 19]. Thus, it is interesting to clarify the inhibitory effects of isoflavones on the reduction of progesterone to 20 α -hydroxyprogesterone in liver cytosol of male rats. However, the inhibitory potency of genistein was much lower than that of apigenin, although these two flavonoids have a planar benzopyrone ring and are the same in the numbers and positions of hydroxyl group. Interestingly, the dihedral angle (31.8°) between the pyrone and phenyl rings of genistein was smaller than that (36.6°) of a more potent inhibitor quercetin, indicating that the molecular conformation of genistein is slightly planar compared to that of quercetin. That is, the lower inhibitory potency of genistein cannot be explained on the basis of a twisted conformation between the pyrone and phenyl rings. It is reasonable to assume that in the case of isoflavones, unlike in the case of other flavonoids such as flavones and flavonols, the position (C3) of the phenyl ring (B ring) is closely associated with the lower inhibitory potencies.

Of flavonoids tested in this study, (+)-catechin was the most poor inhibitor. Recently, a planar catechin analogue has been synthesized from (+)-catechin [13]. Unexpectedly, this catechin analogue

was a poor inhibitor similar to (+)-catechin. As shown in Fig. 6, the molecular conformation of the catechin analogue, like that of (+)-catechin, is puckered due to the saturation of the C2-C3 double bond in the C ring. Furthermore, the two methyl groups shown at arrows in Fig. 6 probably interfere with insertion of the catechin analogue to planar inhibitor-binding cavity on 20 α -HSD that catalyzes the reduction of progesterone to 20 α -hydroxyprogesterone in liver cytosol of male rats. These results suggest that the catechin analogue is not planar compared to apigenin and quercetin, and therefore cannot exhibit potent inhibitory effect.

It has been reported that the scavenging and antioxidative effects of flavonoids play a role in enzyme inhibition [20–23]. For example, because myricetin with a pyrogallol moiety acts as a scavenger of active oxygens or as an antioxidant, it is the most potent inhibitor of succinoxidase [20]. The catechin analogue synthesized from (+)-catechin, despite the absence of C2-C3 double bond, has a radical-scavenging ability comparable to quercetin [24]. However, the inhibitory potency of the catechin analogue was similar to that of (+)-catechin as described above. Thus, it is likely that the inhibition of progesterone reduction to 20 α -hydroxyprogesterone by flavonoids including the catechin analogue does not result from their scavenging or antioxidative effect.

In conclusion, the present study provides evidence to characterize the conversion of progesterone into 20 α -hydroxyprogesterone in rat liver. Furthermore, energy-minimized molecular models demonstrate that a planar A-C-B ring structure is a major factor determining the inhibitory effects of flavonoids other than isoflavones on the reduction of progesterone to 20 α -hydroxyprogesterone in rat liver.

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Figure legends:

Fig. 1. Structures of flavonoids used in this study. The numbers are hydroxylation pattern. rha, rhamnose; ruti, rutinose; glu, glucose.

Fig. 2. HPLC profiles for reduced products of progesterone in liver cytosol and microsomes of male rats. The peaks α and β correspond to authentic 20α -hydroxyprogesterone and 20β -hydroxyprogesterone, respectively.

Fig. 3. Sex difference in the reduction of progesterone to 20α -hydroxyprogesterone (20α -HP) in liver cytosol of rats. Each bar represents the mean \pm S.D. of three rats.

Fig. 4. Effects of various flavonoids on the reduction of progesterone to 20α -hydroxyprogesterone in liver cytosol of male rats. The concentration of inhibitors (flavonoids) was $30\ \mu\text{M}$. Each bar represents the mean \pm S.D. of 4–6 experiments.

Fig. 5. Molecular conformations of apigenin (A), naringenin (B), quercetin (C) and morin (D). These flavonoids are illustrated as the chemical and energy-minimized structures.

Fig. 6. Molecular conformations of (+)-catechin (A) and a planar catechin analogue (B). These flavonoids are illustrated as the chemical and energy-minimized structures. Two arrows show the methyl groups of the catechin analogue.