Characteristics and inhibition by flavonoids of 20α-hydroxysteroid dehydrogenase activity in mouse tissues

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Abstract

Progesterone was stereoselectively reduced to a metabolite 20α-hydroxy-4-pregnen-3-one in the cytosolic fraction from the liver of male mice, indicating that the reduction of progesterone is catalyzed by 20α-hydroxysteroid dehydrogenase (20α-HSD). The cytosolic 20α-HSD activity was observed not only in the liver, but also in the kidney and lung. In liver cytosol, both NADPH and NADH were effective as cofactors for 20α-HSD activity, although NADPH was better than NADH for the enzyme activity. On the other hand, 20α-HSD activity in kidney cytosol required only NADPH as a cofactor. No significant sex-related difference of 20α-HSD activity was observed in liver and kidney cytosols. Flavonoids have been reported to inhibit the biosynthesis and metabolism of steroids. However, little is known about inhibitory effects of flavonoids on 20α-HSD activity. Thus, the effects of 16 flavonoids on 20α-HSD activity were examined, using liver cytosol of male mice. Among flavonoids tested, fisetin, apigenin, naringenin, luteolin, quercetin and kaempferol exhibited high inhibitory potencies for the 20α-HSD activity. We propose the possibility that these flavonoids augment progesterone signaling by inhibiting potently 20α-HSD activity in non-reproductive tissues.

Keywords: 20α-Hydroxysteroid dehydrogenase; Progesterone; Mouse tissues; Cofactor requirement; Flavonoids; Inhibitory potency

Introduction

Progesterone is essential for the initiation and maintenance of pregnancy in mammals. However, progesterone at high levels has some adverse effects on the development of fetuses. For example, if progesterone is injected in large quantities into the amniotic sacs of fetuses or subcutaneously to pregnant mice, it causes fetal death (Petrelli and Forbes, 1964). It has been also reported that progesterone is produced from pregnenolone in non-reproductive tissue such as the rat kidney (Dalla Valle et al., 2004). Furthermore, progesterone and its metabolites have been demonstrated to be a causative factor for breast cancer in women (Ji et al., 2004; Wiebe et al., 2005), although it is required for the full proliferative activity of the breasts.

20α-Hydroxysteroid dehydrogenase (20α-HSD) catalyzes the stereoselective reduction of progesterone to a metabolite 20α-hydroxy-4-pregnen-3-one (20α-HP) and is involved in regulating the amount of progesterone that binds to its nuclear receptor. Thus, 20α-HSD present in the placenta can protect the fetus from the cytotoxic effects of progesterone (Jayasekara et al., 2005). Most mammalian HSDs known thus far belong to either of the aldo-keto reductase (AKR) or short-chain dehydrogenase/reductase (SDR) superfamily (Hyndmann et al., 2003; Oppermann et al., 2003): 20α-HSD is an enzyme belonging to AKR superfamily. Recently, genes encoding mouse, rat, rabbit, goat and human 20α-HSD have been cloned [Akr1c18 (mouse), Akr1c8 (rat), Akr1c5 (rabbit), Akr1c1 (human)] (Ishida et al., 1999; Miura et al., 1994; Lacy et al., 1993; Zhang et al., 2000; Nishizawa et al., 2000; Jayasekara et al.,

2004). In addition, the tissue expression patterns of 20α-HSD mRNA have been shown in the case of the mouse enzyme (AKR1C18) (Ishida et al., 1999). That is, the expression of 20α-HSD mRNA is prominent in the ovary during pregnancy. However, the enzyme activity patterns and characterizations of 20α-HSD present in non-reproductive tissues of mice remain to be elucidated.

Our previous paper (Shimada et al., 2005) has pointed out that flavonoids such as apigenin and luteolin potently inhibit 20α-HSD activity, namely the reduction of progesterone to 20α-HP, in rat liver. It is possible that flavonoids, which are widely distributed in vegetables, fruits and green tea, disturb the homeostasis of progesterone and its metabolite levels in various tissue cells. The purpose of the present study was to examine characteristics of 20α-HSD activity in mouse non-reproductive tissues. We also attempted to evaluate the inhibitory effects of flavonoids on 20α-HSD activity in mouse liver.

Materials and methods

Chemicals

Progesterone (4-pregnene-3,20-dione), 20α-hydroxy-4-pregnen-3-one (20α-HP) and 20β-hydroxy-4-pregnen-3-one (20β-HP) were purchased from Sigma (St. Louis, MO). Flavonoids were obtained from the following sources: morin, myricetin, genistein, taxifolin (racemate), kaempferol and daidzein (Sigma); naringenin, apigenin and (-)-epicatechin (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). 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.

Animals

Male and female ddY mice at 9–11 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.

Preparation of subcellular fractions

Animals were slightly 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.

Assay of 20α- and 20β–HSD activities

The enzyme activities in subcellular fractions were 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), $MgCl_2$ (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), $MgCl_2$ (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 (20α-HP and 20β-HP) of progesterone were determined by HPLC according to a slightly modified of Swinney et al (1987). 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. Protein concentration was estimated by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

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 final concentration of flavonoids tested was 20 μM. The IC_{50} 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.

Statistical analysis

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

Results

20α-HSD activity in subcellular fractions from the liver of male mice

The reduction of progesterone to 20α-HP or 20β-HP was examined in liver cytosol and microsomes of male mice. As shown in Fig. 1, 20α-HP was formed in the cytosolic reaction system and appeared as a main peak in the HPLC profile, whereas 20β-HP was not detected. In the microsomal reaction system, neither 20α-HP nor 20β-HP was detected (data not shown). These results indicate that in the liver of male mice, cytosolic 20α-HSD plays an important role in the stereoselective reduction of progesterone.

20α-HSD activity in cytosolic fractions from various tissues of male mice

Figure 2 shows 20α-HSD activities in cytosolic fractions from various tissues of male mice. The liver exhibited the highest 20α-HSD activity. The kidney and lung also exhibited significant 20α-HSD activities. However, little 20α-HSD activity was detected in the brain and heart.

Cofactor requirement and sex difference for 20α-HSD activity

The cofactor requirement for 20α-HSD activity was examined in cytosolic fractions from the liver and kidney of male mice. In liver cytosol, both NADPH and NADH were effective as cofactors for 20α-HSD activity, although NADPH was better than NADH for the enzyme activity (Fig. 3A). On the other hand, 20α-HSD activity in kidney cytosol required only NADPH as a cofactor (Fig. 3B). Furthermore, we attempted to elucidate sex difference of 20α-HSD activity in cytosolic fractions from the liver and kidney of mice. As shown in Fig. 4, no significant sex-related difference of 20α-HSD activity was observed in liver and kidney cytosols.

Inhibition of 20α-HSD activity by flavonoids

We investigated the inhibitory effects of 16 flavonoids listed in Fig. 5 on 20α-HSD activity in liver cytosol of male mice. Figure 6 shows the inhibition percentages of flavonoids at a concentration of 20 μM. Furthermore, the IC_{50} values are summarized in Table 1. Among these flavonoids, fisetin, apigenin, naringenin, luteolin, quercetin and kaempferol exhibited high inhibitory potencies. The inhibitory potencies of

quercitrin, (-)-epicatechin, (+)-catechin and genistin were much lower than those of other flavonoids.

Discussion

The present study provided evidence that progesterone is stereoselectively reduced to 20α-HP in the cytosolic fractions from the liver, kidney and lung of male mice. It should be noted that 20α-HSD activity is detectable in non-reproductive tissues such as the liver, kidney and lung. Furthermore, Vergnes et al. (2003) have previously shown that high expression of mouse 20α-HSD gene (Akr1c18) is observed not only in the ovary, but also in the kidney, lung and spleen. 20α-HSD may protect these non-reproductive tissues from adverse effects of progesterone by converting to a metabolite 20α-HP.

20α-HSD belongs to AKR superfamily according to the protein phylogeny (Penning, 1997). Members of this family, unlike those of SDR superfamily, prefer NADPH as a cofactor in the process of reductive metabolism (Jörnvall et al., 1995). In the present study, however, both NADPH and NADH were effective for the reduction of progesterone to 20α-HP in liver cytosol, even though NADPH was better than NADH for the enzyme activity. NADH was without effect in kidney cytosol. Based on these results, it seems likely that NADH-dependent enzyme(s) other than 20α-HSD contributes in part to the reduction of progesterone to 20α-HP in liver cytosol.

It has been reported that in non-reproductive tissues of mice, the expression of

20α-HSD mRNA is higher in the female than in the male (Pelletier et al., 2003). In the present study, however, there is no significant sex-related difference of 20α-HSD activity in liver cytosol. A similar result was observed for the enzyme activity in kidney cytosol. This may be because several AKR enzymes including AKR1C18 (mouse 20α-HSD) exhibit NADPH-dependent 20α-HSD activity in the cytosolic fractions of mouse liver and kidney, although further studies should be conducted.

Furthermore, we examined the structural characteristics of flavonoids necessary for inhibiting NADPH-dependent 20α-HSD activity in liver cytosol of male mice. The enzyme activity was potently inhibited by a variety of flavones, flavonols and flavanone (naringenin). In particular, it should be noted that the IC_{50} value of fisetin is much smaller than that of other flavonoids. However, glycosylated flavonol (quercitrin), glycosylated isoflavone (genistin) and catechins were poor inhibitors. Our previous study (Shimada et al., 2005) has shown that the double bond of 2- and 3-position (C2-C3) in the C ring of flavonoids plays an important role in their inhibitory potencies for NADPH-dependent 20α-HSD activity in liver cytosol of male rats. For example, the IC_{50} values of apigenin and naringenin were 8.8 ± 0.8 and > 50 μM, respectively (Shimada et al., 2005): apigenin and naringenin are the same in the numbers and positions of hydroxyl group, except the presence or absence of the C2-C3 double bond in the C ring. In the present study using liver cytosol of male mice, however, apigenin (IC_{50} = 3.0 ± 0.6 μM) and naringenin (IC_{50} = 3.3 ± 0.3 μM) exhibited a similar inhibitory potency for 20α-HSD activity. Thus, the inhibitory potencies of flavonoids for 20α-HSD activity in liver cytosol of male mice cannot be explained on the basis of

the C2-C3 double bond in the C ring. Additional studies are in progress to elucidate mechanism for the inhibition of 20α-HSD activity in liver cytosol of male mice by flavonoids.

Flavonoids can protect against oxidative stress by scavenging reactive oxygen intermediates (Jovanovic et al., 1994; Hanasaki et al., 1994; Hu et al., 1995). Since oxidative damage to biomolecules, such as DNA, proteins and polyunsaturated fatty acids, is thought to cause cancer and aging, considerable attention has been focused on the development of antioxidants to treat diseases associated with oxidative stress. However, there is also evidence that flavonoids are mutagenic and carcinogenic in both bacterial and mammalian experimental systems (Ochiai et al., 1984; Rueff et al., 1986; Pamukcu et al., 1980). Piekorz et al. (2005) have recently demonstrated that in female mice, Stat5 (transcription factor) plays a critical role in suppressing 20α-HSD during pregnancy and that the primary function of 20α-HSD is to regulate progesterone levels at term of pregnancy. Furthermore, selective loss of human 20α-HSD (AKR1C1) has been observed for tissue samples of breast cancer in women (Ji et al., 2004). 20α-HSD in normal tissue may regulate progesterone-dependent gene expression by limiting interaction with its nuclear receptor. In the present study, a variety of flavonoids were found to inhibit potently 20α-HSD activity in liver cytosol of male mice. We propose the possibility that flavonoids such as fisetin and apigenin augment progesterone signaling by inhibiting potently 20α-HSD activity in reproductive and non-reproductive tissues.

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Figure legends:

Fig. 1. HPLC profile for the reduction product of progesterone in liver cytosol of male mice. (A) reduction product of progesterone; (B) authentic samples. The peak p, α and β correspond to authentic progesterone, 20α-hydroxy-4-pregnen-3-one (20α-HP) and 20β-hydroxy-4-pregnen-3-one (20β-HP), respectively.

Fig. 2. 20α-HSD activity in cytosolic fractions from various tissues of male mice. Progesterone at a concentration of 0.1 mM was used as the substrate. Each bar represents the mean ± S.D. of three experiments.

Fig. 3. Cofactor requirement for 20α-HSD activity in liver (A) and kidney (B) cytosols of male mice. Progesterone (0.1 mM) as the substrate and NADPH, NADH, NADP and NAD (0.5 mM) as the cofactors were used. Each bar represents the mean ± S.D. of three experiments. **P < 0.01, significantly different from NADH in liver cytosol.

Fig. 4. Sex difference for 20α-HSD activity in liver (A) and kidney (B) cytosols of mice. Progesterone at a concentration of 0.1 mM was used as the substrate. Each bar represents the mean ± S.D. of three experiments.

Fig. 5. Chemical structures of flavonoids used in this study. The numbers are hydroxylation pattern. rha, rhamnosyl; ruti, rutinosyl; glu, glucosyl.

Fig. 6. Inhibition of 20α-HSD activity in liver cytosol of male mice by flavonoids. Progesterone at a concentration of 0.1 mM was used as the substrate. The concentration of inhibitors (flavonoids) was 20 μM. Each bar represents the mean ± S.D. of three experiments.