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Bovine adrenal 3β-hydroxysteroid dehydrogenase (E.C. 1.1.1.145)/5-ene-4-ene isomerase (E.C. 5.3.3.1): characterization and its inhibition by isoflavones

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Abstract

The isoflavones daidzein, genistein, biochanin A and formononetin inhibit potently and preferentially the γ-isozymes of mammalian alcohol dehydrogenase (γγ-ADH), the only ADH isozyme that catalyzes the oxidation of 3β-hydroxysteroids. Based on these results, we proposed that these isoflavones might also act on other enzymes involved in 3β-hydroxysteroid metabolism. Recently, we showed that they indeed are potent inhibitors of a bacterial β-hydroxysteroid dehydrogenase (β-HSD). To extend this finding to the mammalian systems, we hereby purified, characterized and studied the effects of isoflavones and structurally related compounds on, a bovine adrenal 3β-hydroxysteroid dehydrogenase (3β-HSD). This enzyme catalyzes the oxidation of 3β-hydroxysteroids but not 3α-, 11β- or 17β-hydroxysteroids. The same enzyme also catalyzes 5-ene-4-ene isomerization, converting 5-pregnen 3, 20-dione to progesterone. The K_m values of its dehydrogenase activity determined for a list of 3β-hydroxysteroid substrates are similar (1 to 2 μM) and that of its isomerase activity, determined with 5-pregnen 3, 20-dione as a substrate, is 10 μM. The k_{cat} value determined for its isomerase activity (18.2 min^{-1}) is also higher than that for its dehydrogenase activity (1.4–2.4 min^{-1}). A survey of more than 30 isoflavones and structurally related compounds revealed that daidzein, genistein, biochanin A and formononetin inhibit both the dehydrogenase and isomerase activity of this enzyme. Inhibition is potent and concentration dependent. IC_{50} values determined for these compounds range from 0.4 to 11 μM, within the plasma and urine concentration ranges of daidzein and genistein of individuals on vegetarian diet or semi-vegetarian diet. These results suggest that dietary isoflavones may exert their biological effects by inhibiting the action of 3β-HSD, a key enzyme of neurosteroid and/or steroid hormone biosynthesis.

1. Introduction

The enzyme complex 3β-hydroxy-5-ene-steroid dehydrogenase (E.C. 1.1.1.145)/steroid Δ^5-Δ^4-ene isomerase (E.C. 5.3.3.1) (3β-HSD/isomerase) catalyzes the conversion of Δ^5-ene-3β-hydroxy steroids to Δ^4-3-ketosteroids (e.g. pregnenolone to progesterone), a two-step pathway involving a dehydrogenase and an isomerase reaction (Fig. 1). In *Pseudomonas* bacteria, these reactions are catalyzed by two distinct enzymes [1]. However, in mammalian tissues, both activities appear to reside in a single enzyme [2]. Thus, dehydrogenase and isomerase activities have been co-purified from human placenta [3], human adrenals [4], rat adrenals [5], rat testis [6] and bovine adrenals [7]. In humans, two 3β-HSD/isomerase isoforms type I and II have been identified with type I isozyme being more active [8]. 3β-HSD/isomerase is found in virtually all tissues studied, present in the adrenals, testes, ovaries, placenta, adipose tissue, prostate, breast, kidney, seminal vesicle,

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uterus, liver, heart, thymus, pancreas, lung, spleen, skin and brain. It plays an important role in biosynthesis of all classes of hormonal steroids including progesterone, mineralocorticoids, glucocorticoids androgens and estrogens. The ontogeny of 3β-HSD expression in human and rat adrenal gland, testis and ovary is closely correlated with steroid hormone biosynthesis [8]. Further, a recent study has shown that 3β-HSD/isomerase is actively expressed in rat brain where the enzyme is believed to play an essential role in the biosynthesis of neurosteroids [9].

Isoflavones are benzo-γ-pyrone derivatives found mostly in leguminous plant cells [10]. They are particularly rich in certain legumes (Soya, lentils, beans etc.) which are components of traditional diets for most Asians, Africans, South and Central Americans, and East and South Europeans, and are believed to have a range of beneficial effects seemingly related to their mild estrogenic/anti-estrogenic activity [11]. Therefore, further evaluation of their biochemical and physiological effects on the metabolism of estrogen and steroid hormones in general, is of significant importance. Isoflavones exhibit a wide spectrum of biochemical and physiological activities such as anti-cancer [12]; anti-angiogenic [13]; estrogenic and anti-estrogenic [14,15]; anti-oxidant [16]; anti-microbial [17]; anti-hypertensive [18]; anti-hyperthermic [19]; amethystic [20]; and anti-dipsotropic activity [21]. They have also been shown to have anabolic effect on bone metabolism [22]; induce leukaemic cells differentiation [23]; and inhibit enzymes involved in regulation of cell growth such as topoisomerase [24], tyrosine protein kinase [25]; and steroid metabolism such as aromatase/estrogen synthetase [11] and 17β-hydroxysteroid oxidoreductase [26].

The isoflavones daidzein, genistein, biochanin A and formononetin selectively inhibit human γγ- but not ββ-ADH in vitro [27]. Since human γγ-ADH is the only ADH isozyme that catalyzes the oxidation of 3β-hydroxysteroids and subjects to β-estradiol inhibition, it seems likely that these compounds might also inhibit β-HSD and exert some of their biological effects via steroidal mechanisms that involve estrogens and/or 3β-hydroxysteroids such as pregnenolone and dehydroepiandrosterone. Using β-hydroxysteroid dehydrogenase (β-HSD) isolated from *P. testosteronii*, we demonstrated that these isoflavones are potent inhibitors of the bacterial enzyme. Indeed, all isoflavones that inhibit γγ-ADH also inhibit bacteria β-HSD very potently [28]. To extend this study to the mammalian systems, we hereby purified, characterized and studied the effects of isoflavones and structurally related compounds on, a bovine adrenal 3β-HSD.

The formation and hydrolysis of sulfoconjugates of hydroxysteroids are controlled by hydroxysteroid sulfotransferase (EC 2.8.2.2) and sterol sulfate sulfohydrolase (EC 3.1.6.2), respectively [29]. Recent studies have shown that sulfoconjugates of steroids and for that matter other biomolecules as well, are not necessarily inert end products of metabolism without physiological significance. Many of them have been found to play strategically important functional roles in the central and peripheral systems [30]. For instance, pregnenolone sulfate is a negative modulator of the $GABA_A$ receptor but acts as a positive modulator on the NMDA receptor [30]. Hence, the sulfoconjugation of pregnanolone and sulfohydrolysis of its sulfoconjugate could play important role in switching on and off a neurone by regulating the activities of the inhibitory (GABA) and stimulatory (NMDA) neuromodulators. Furthermore, sulfoconjugates of estrone and dehydroepiandrosterone serve not only as water soluble excretion products of the respective steroids but also as their inactive transport form in the circulation. In fact, in postmenopausal women, in whom breast cancer most frequently occurs, biologically active estrogenic steroids in mammary tissue are derived almost exclusively from their inactive sulfoconjugates — estrone sulfate and dehydroepiandrosterone sulfate — via the peripheral sterol sulfatase pathway [31]. Blocking the local supply of biologically active estrogens and

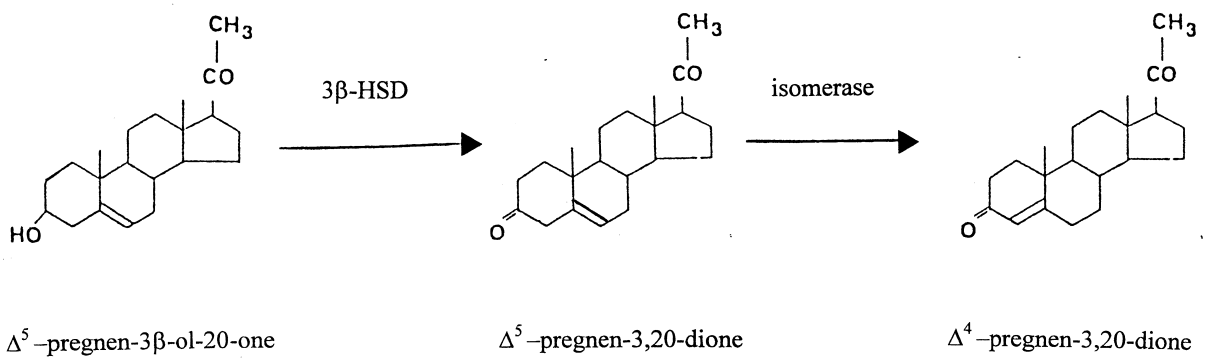

Fig. 1. Conversion of Δ^5-pregnen-3β-ol-20-one (pregnenolone) to Δ^4-pregnen-3,20-dione (progesterone) via the metabolic intermediate Δ^5-pregnen-3,20-dione catalyzed by 3β-HSD/isomerase.

androstendiol in the breast by modulating activities of enzymes involved in sulfoconjugation and sulfohydrolysis of steroids is actively persued as a strategy for the development of new treatments and preventive measures for breast cancer. Therefore, the effects of the isoflavone daidzein on hydroxysteroid sulfotransferase and steroid sulfatase activities were also investigated using partially purified hamster liver enzymes.

2. Materials and methods

2.1. Materials

Adrenals of mature bovine were purchased from Pel-Freez (Rogers, AR). Steroids were from Steraloids, Inc. (Wilton, NH). Hamsters were from Sasco, Omaha, NE. Hamsters were sacrificed by decapitation under ether anathema and livers and adrenals were removed and stored at −70°C. Isoflavones were products of Indofine Chemical Co. Inc. (Somerville, NJ). DTT was obtained from United State Biochemicals (Cleveland, OH). Methanol, chloroform and toluene purchased from J.T. Baker (Phillipsburg, NJ) and Fisher (Pittsburgh, PA) were HPLC grade. Glycerol, NAD^+, EDTA, adenosine 5′-monophosphate Sepharose 4B, adenosine 3′-phosphate 5′-phosphosulfate (PAPS) were from Sigma Chemical Co. (St. Louis, MO). DE-52 was product of Whatman (Hillsboro, OR). Bio-Gel hydroxyapatite and Tris–HCl ready gel, 4–20%, were obtained from Bio-Rad (Hercules, CA). PD-10 columns were purchased from Pharmacia (Uppsala, Sweden). YM10 ultrafiltration membrane was from Amicon (Beverly, MA). [1,2,6,7-^{3}H(N)]-dehydroepiandrosterone and [7-^{3}H(N)]-dehydroepiandrosterone sulfate, sodium salt, were products of Dupont NEN (Boston, MA). Hydrofluor and Ecoscint O scintillation solution for aqueous and non-aqueous radioactive samples were from National Diagnostics (Manville, NJ). All other chemicals are of reagent grade quality.

2.2. Purification of bovine adrenal microsomal 3β-HSD/isomerase

Bovine adrenal microsomal 3β-HSD/isomerase was purified according to the method described for the rat enzyme with slight modification [5]. Briefly, microsomal fraction was first obtained from adrenal gland by homogenization followed by centrifugation. The microsomes were solubilized and the enzyme was purified by chromatography on DE-52, hydroxylapatite and AMP-sepharose 4B columns. The minimal molecular weight and purity of the purified enzyme were determined on SDS-polyacrylamide gel electrophoresis [32].

2.3. Preparation of hamster liver cytosolic fraction

Hamster liver was coarsely minced and homogenized in 10 mM triethanolamine (pH 7.5) containing 1.5 mM DTT and 20% glycerol. The homogenate was centrifuged at 12,000 *g* for 15 min and the supernatant was further centrifuged at 100,000 *g* for 1 h to obtain the cytosolic fraction. Salt and other small molecules were removed by passing through a PD-10 column in homogenizing buffer. The resulting cytosolic fraction was diluted with the homogenizing buffer to a proper concentration so that each ml of enzyme catalyzed the formation of a nmol of product per min under the assay conditions described below.

2.4. Preparation of hamster liver and adrenal microsomal fraction

Liver or adrenal tissues were coarsely minced and then homogenized in 1 volume of ice-cold 0.25 M sucrose. The homogenate was centrifuged at 12,500 *g* for 20 min at 4°C and the supernatant was further centrifuged at 105,000 *g* for 1 h. The pellet which contained sterol sulfatase activity was washed three times in 0.05 M Tris–HCl (pH 7.6) and was suspended in the same buffer containing 1% triton X-100. The suspension was sonicated 10 cycles each with 15 s bursts of 100 W sonic wave (Branson Sonifier 450, VMR Scientific). Insoluble material was removed by centrifugation at 105,000 *g* for 90 min. The supernatant which contain sterol sulfatase activity was desalted on a PD-10 column in suspension buffer and was used without further purification. Protein concentrations were determined by the method of Bradford [33] using bovine serum albumin as the standard.

2.5. 3β-HSD/isomerase assay

The 3β-HSD activity was measured spectrophotometrically at 25°C in a standard 1 ml assay medium containing 100 mM KPi (pH 7.5), 20% glycerol, 1% MeOH, 0.1 mM EDTA, 1 mM NAD^+ and 0.1 mM dehydroepiandrosterone (DHEA). The reaction were monitored at 340 nm ($\in_{NADH} = 6.22\ mM^{-1}\ cm^{-1}$) in a Varian Cary 1E UV-visible spectrophotometer [5].

The steady state kinetic constants of 3β-HSD activity were determined from double reciprocal plots derived from initial velocities measured at five different substrate concentrations (0.5 to 8 × K_m). Under these conditions, 3β-HSD catalyzed oxidation of the hydroxysteroid substrates followed normal Michaelis–Menten kinetics.

The isomerase activity were measured in a 1 ml assay medium containing 100 mM KPi (pH 7.5), 20% glycerol, 0.1 mM EDTA, 0.1 mM NAD^+, 0.025 nM 3β-HSD/isomerase, 2% MeOH and various concen-

trations of the substrate 5-pregnen 3,20-dione. Isomerase reaction rates were followed at 248 nm ($\in_{\text{progesterone}} = 16.3\ \text{mM}^{-1}\ \text{cm}^{-1}$) at 25°C with Varian Cary 1E UV-visible spectrophotometer at 25°C. Controls for correcting spontaneous isomerization were run in parallel [34]. The steady state kinetic constants of isomerase activity were determined from double reciprocal plots derived from initial velocities measured at 5 different substrate concentrations (0.05 to 0.8 × K_m). Reaction rates at substrate concentration $\geq K_m$ were not determined because of poor solubility of the substrate. Under these conditions, the substrate saturation curves followed normal Michaelis–Menten kinetics.

2.6. Enzyme inhibition assay

Inhibition of 3β-HSD and isomerase activities by isoflavones and related compounds were measured under the assay conditions similar to those described above except that various concentrations of test compounds were added. Enzyme inhibition was determined at substrate concentrations equaled to K_m and expressed as % inhibition$= [(v_o - v_i)/v_o] \times 100$, where v_o and v_i are the enzyme reaction rates measured in the absence and presence of an inhibitor, respectively. IC_{50} values are inhibitor concentrations at which reaction rates were suppressed by 50%.

2.7. Hydroxysteroid sulfotransferase assay

Hydroxysteroid sulfotransferase was measured according to the method of Falany et al. with slight modification [35]. Assays were performed in a 250 μl pH 7.5 assay medium containing 50 mM Tris–HCl, 10 mM $MgCl_2$, 1 μM ^{3}H-DHEA (0.4 μCi nmol^{-1}), 20 μM PAPS and an aliquot of hamster liver cytosol (150 μg protein). Reaction was allowed to proceed for 5 min at 37°C and terminated by the addition of 3 ml chloroform and 0.25 ml of 0.25 M Tris–HCl, pH 8.7. The radioactive reaction product, ^{3}H-DHEA-sulfate, was extracted into the aqueous phase and quantitated in a liquid scintillation counter.

2.8. Steroid sulfatase assay

The method of Suzuki et al. [36] was followed with slight modification. Steroid sulfatase activity was assayed in a 350 μl pH 7.5 assay medium containing 50 mM Tris–HCl, 4 μM ^{3}H-DHEA sulphate (0.07 μCi/nmol) and steroid sulfatase preparation (100 μg protein). Reaction was allowed to proceed for 20 min at 37°C and terminated by the addition of 0.35 ml of 0.1 N NaOH and 1 ml toluene. The radioactive reaction product, ^{3}H-DHEA, was extracted into the organic phase and quantitated in a liquid scintillation counter.

3. Results

3.1. Effect of daidzein on partially purified hamster liver cytosolic hydroxysteroid sulfotransferase, microsomal steroid sulfatase and adrenal microsomal 3β-HSD

Daidzein inhibits both the hamster and bovine adrenal 3β-HSD activity in the crude microsomal fractions. Inhibition is concentration dependent and the IC_{50} values determined for both the hamster and bovine enzymes are ~1 μM. On the other hand, daidzein, at a concentration up to 20 μM, has little, if any, effect on the hydroxysteroid sulfotransferase and steroid sulfatase activities in the crude hamster liver cytosolic fractions (Table 1). Therefore, only adrenal 3β-HSD/isomerase was purified to homogeneity and its inhibition by daidzein and its structurally related compounds were further investigated. Bovine instead of the hamster adrenal was used as the source of 3β-HSD/isomerase because the former is abundant and commercially available. Furthermore, the bovine enzyme is equally sensitive to daidzein inhibition as its hamster counterpart.

3.2. Purification and characterization of bovine adrenal 3β-HSD/isomerase

The bovine adrenal 3β-HSD/isomerase purified in our laboratory migrates as a single protein band upon SDS-PAGE. Its apparent molecular weight is 41,000 similar to those reported in the literature [37,38]. The purified enzyme exhibits both 3β-HSD and 5-ene-4-ene isomerase activity, catalyzes the conversion of Δ^5-pregnen-3β-ol-20-one (pregnenolone) to Δ^4-pregnen-3,20-dione (progesterone) via the metabolic intermediate Δ^5-pregnen-3,20-dione (Fig. 1). Using pregnenolone and Δ^5-pregnen-3,20-dione as the substrates, the specific activities of the 3β-HSD and isomerase activity of the purified enzyme were determined to be 29.3 and 218 nmol min^{-1} mg^{-1}, respectively.

The substrate specificity of the 3β-HSD activity of the purified enzyme was examined with a list of hydroxysteroid substrates. The bovine enzyme catalyzes the oxidation of all 3β-hydroxysteroids studied, regardless of their configuration at the 5 position. Hence Δ^5-pregnen-3β-ol-20-one, 5α-pregnan-3β-ol-20-one, 5β-pregnan-3β-ol-20-one, 5α-androstan-3β-ol-17-one, 5β-androstan-3β-17β-diol, Δ^5–androsten-3β,17β-diol and Δ^5–androsten-3β-ol-17-one are all substrates for this enzyme, whereas the 3α-, 11β-, 17β-, 20α-, 21-hydroxysteroids such as 5β-pregnan-3α-20α-diol, 5α-androstan-3α-ol-17-one, 5β-androstan-3α-ol-17-one, 4-pregnen-

Table 1
Effect of daidzein on the activity of partially purified 3β-HSD, hydroxysteroid sulfotransferase and steroid sulfatase[a]

Daidzein (μM)	Activity, % of control			
	hamster adrenal microsomal 3β-HSD	hamster liver cytosolic hydroxysteroid sulfotransferase	hamster liver microsomal steroid sulfatase	bovine adrenal microsomal 3β-HSD
0	100	100	100	100
1	54	ND	ND	50
2	ND	100	100	ND
10	25	100	100	35
20	ND	100	100	ND

[a] 3β-HSD, hydroxysteroid sulfotransferase and steroid sulfatase were assayed in duplicates according to the procedures described in Materials and Methods using pregnenolone (25 μM), ^{3}H-DHEA (1 μM) and ^{3}H-DHEA sulfate (4 μM) as the substrates, respectively. ND = not determined.

11β, 21-diol-3,20-dione, 4-androsten-17β-ol-3-one are not substrates for the enzyme (Table 2).

The K_m values obtained for the 3β-HSD activity of the bovine adrenal enzyme using different 3β-hydroxysteroids are very similar, ranging from 1 to 2 μM (Table 3). The K_m value of the isomerase activity of the enzyme for Δ^5-pregnen 3,20-dione is 10 μM. The turnover number of 3β-HSD towards various 3β-hydroxysteroids are also very similar, ranging from 1.4–2.4 min^{-1}. The turnover number of the isomerase activity is 18.2 min^{-1}, much higher than that of 3β-HSD activity (Table 3).

3.3. Inhibition of bovine adrenal 3β-HSD/isomerase by isoflavones and other related compounds

Survey of structurally related isoflavones discovered four compounds that inhibit 3β-HSD activity potently. Among which, biochanin A is most potent (IC_{50} = 0.5 μM) followed by genistein (1.5 μM), daidzein and formononetin (3.7 μM). Puerarin, prunetin and apigenin also inhibit 3β-HSD but only at high concentration ($IC_{50} \gg 10$ μM) (Table 4). Most of the commercially available isoflavonoid, flavonoid, flavan and coumarin compounds tested do not inhibit β-HSD at concentrations up to 10 μM (Table 5).

The 17β-estradiol also inhibits 3β-HSD activity very potently. However, phytoestrogens 18α-glycyrrhetinic acid, β-sitosterol and zearalenone have no effect on 3β-HSD activity. Daidzein also inhibits the reductase activity of 3β-HSD with an IC_{50} value (9.2 μM) slightly higher than that determined for its dehydrogenase activity (Table 4).

Besides pregnenolone, oxidation of other 3β-hydroxysteroids such as androstenediol, DHEA and 5α-pregnan-3β-ol-20-one catalyzed by 3β-HSD/isomerase is also potently inhibited by daidzein, biochanin A, genistein and formononetin (Table 6). It appears that the inhibition is most potent with and 5α-pregnan-3β-

Table 2
Substrate specificity of bovine adrenal 3β-HSD[a]

Steroid substrate	Activity nmol min^{-1}
Δ^5-pregnen-3β-ol-20-one (pregnenolone)	0.19
Δ^5-androsten-3β,17β-diol (androstenediol)	0.15
Δ^5-androsten-3β-ol-17-one (dehydroepiandrosterone, DHEA)	0.16
5α-pregnan-3β-ol-20-one (allopregnanolone)	0.23
5α-androstan-3β-ol-17-one	0.24
5β-pregnan-3β-ol-20-one	0.31
5β-androstan-3β-17β-diol	0.13
5β-pregnan-3α,20α-diol	NA
5α-androstan-3α-ol-17-one	NA
5β-androstan-3α-ol-17-one	NA
5β-androstan-3α,17β-diol	NA
4-pregnen-11β, 21-diol-3,20-dione (corticosterone)	NA
1,3,5[10]-estratriene-3,17β-diol (17β-estradiol)	NA
5β-pregnan-3α-ol-20-one	NA
Δ^4-androsten-17β-ol-3-one (testosterone)	NA

[a] 3β-HSD activities were assayed in triplicates at 25°C in a standard 1 ml pH 7.5 assay medium containing 100 mM KPi, 20% glycerol, 0.1 mM EDTA, 2% MeOH, 0.8 mM NAD^+, 0.1 nmol enzyme and 10 μM steroids. NA = not a substrate.

Table 3
Kinetic parameters of 3β-HSD[a] and 5-ene-4-ene isomerase[b] activity of bovine adrenal 3β-HSD/isomerase (ND = not determined)

Substrate	K_m (μM)		k_{cat} (min^{-1})	
	3β-HSD	Isomerase	3β-HSD	Isomerase
Δ^5-pregnen-3β-ol-20-one (pregnenolone)	2.0	ND	2.4	ND
Δ^5-androsten-3β,17β-diol (androstenediol)	1.0	ND	2.0	ND
Δ^5-androsten-3β-ol-17-one (DHEA)	1.0	ND	2.0	ND
5α-pregnan-3β-ol-20-one (allopregnanolone)	1.0	ND	1.4	ND
Δ^5-pregnen-3,20-dione	ND	10	ND	18.2

[a] 3β-HSD activities were assayed in triplicates at 25°C in a standard 1 ml pH 7.5 assay medium containing 100 mM KPi, 20% glycerol, 0.1 mM EDTA, 2% MeOH, 0.8 mM NAD^+, 0.1 nmole enzyme and various concentrations of steroid substrates.
[b] Isomerase activities were assayed in triplicates at 25°C in a standard 1 ml pH 7.5 assay medium containing 100 mM KPi, 20% glycerol, 0.1 mM EDTA, 2% MeOH, 0.1 mM NAD^+, 0.025 nmole enzyme and various concentrations of Δ^5-pregnen 3,20-dione.

ol-20-one as the substrate but less so with androstenediol and DHEA as substrates.

The isomerase activity of 3β-HSD/isomerase is also inhibited by daidzein, formononetin, genistein, biochanin A and 17β-estradiol (Table 7). The IC_{50} values of these compounds determined at 10 μM of Δ^5-pregnen-3,20-dione (K_m of isomerase) range from 0.3 to 4 μM. Other isoflavones and structurally or functionally related compounds surveyed do not inhibit 5-ene-4-ene isomerase activity of this enzyme (Table 7).

4. Discussion

Substrate specificity studies of bovine adrenal 3β-HSD/isomerase indicated that it oxidizes both 3β-hydroxy-5α- and 3β-hydroxy-5β-steroids (Table 2). On the contrary, the substrate specificity of the hydroxysteroid oxidative activity of human liver γγ-ADH is more restrictive. It only catalyzes the oxidation of the 3β-hydroxy function of hydroxysteroids that have a β-configuration at the 5-position such as 3β-hydroxy-5β-androstan-17-one, 3β-hydroxy-5β-pregnan-20-one, 3β-hydroxyetiocholanic acid, 5β,20(22)-cardenolide-3β,14-diol (digitoxigenin). Hydroxysteroids with a 3α-hydroxy-5β-, 3α-hydroxy-5α-, or 3β-hydroxy-5α- configurations are not substrate of γγ-ADH [39]. On the other hand, the bacterial β-HSD is more accommodating than the bovine adrenal 3β-HSD/isomerase, catalyzing the oxidation of not only the 3β-hydroxy function of the hydroxysteroids of the C_{19} and C_{21} series, but also the 16β- and 17β-hydroxy groups of hydroxysteroids of the C_{18}, C_{19} and C_{21} series. Like the bovine 3β-HSD and human γ-ADH, the bacterial enzyme does not catalyzes the oxidation of 3α- or 17α-hydroxy group of selected hydroxysteroid substrates [28]. The K_m values for 3β-HSD and isomerase activities of bovine 3β-HSD/isomerase determined in this study were similar to those reported for the human placental enzyme [3].

Table 4
Daidzein and structurally or functional related compounds that inhibit bovine adrenal 3β-HSD[a]

Type	Substituents							Name	IC_{50} (μM)
	2	3	4	5	7	8	4′		
Isoflavone	H		=O	H	OH	H	OH	Daidzein	3.7
	H		=O	H	OH	H	OH	Daidzein	9.2[b]
	H		=O	OH	OH	H	OH	Genistein	1.5
	H		=O	H	OH	H	OMe	Formononetin	3.7
	H		=O	OH	OH	H	OMe	Biochanin A	0.5
	H		=O	H	OH	CGlc	OH	Puerarin	≫10
	H		=O	OH	OMe	H	OH	Prunetin	≫10
Flavone	H		=O	OH	OH	H	OH	Apigenin	≫10
Estrogen								17β-Estradiol	0.9

[a] 3β-HSD activities were assayed in triplicates at 25°C in a standard 1 ml pH 7.5 assay medium containing 100 mM KPi, 20% glycerol, 0.1 mM EDTA, 2% MeOH, 2 μM pregnenolone, 0.8 mM NAD^+, 0.1 nmol enzyme and various concentrations of inhibitors. IC_{50} values are inhibitor concentrations at which enzyme reaction rates were suppressed by 50%.
[b] IC_{50} value for daidzein inhibition of the reductase activity of 3β-HSD. Reductase activity was assayed in triplicates at 25°C in a standard 1 ml pH 7.5 assay medium containing 100 mM KPi, 20% glycerol, 0.1 mM EDTA, 2% MeOH, 0.1 mM NADH, 0.1 nmole enzyme and 2 μM of Δ^5-pregnen-3,20-dione as the substrate.

Table 5
Isoflavones, flavones and other functional related compounds that do not inhibit bovine adrenal 3β-HSD[a]

Type	Substituent											Name
	2	3	4	5	6	7	8	2′	3′	4′	5′	
Isoflavone												
	H		=O	H	H	OGlc	H	H	H	OMe	H	ononin
	H		=O	H	H	H	H	H	H	I-Pr	H	4′isopropyl-isoflavone
	H		=O	OH	H	OGlc	H	H	H	OH	H	genistin
	H		=O	H	H	OGlc	H	H	H	OH	H	daidzin
Flavone												
		H	=O	H	H	H	H	H	H	H	H	flavone
		H	=O	H	H	OH	H	H	H	H	H	7-hydroxyflavone
		H	=O	H	H	H	H	Cl	H	H	H	2′-chloroflavone
		H	=O	H	H	O_2CPh	H	H	H	H	H	7-benzoyl-oxyflavone
		H	=O	H	H	OH	OH	H	H	H	H	7,8-dihydroxy-flavone
		H′	=O	OH	H	OH	H	H	H	H	H	chrysin
		OH	=O	H	H	H	H	H	H	H	H	3-hydroxyflavone
		OH	=O	OH	H	OH	H	H	H′	H	H	galangin
		OH	=O	H	H	OH	H	H	OH	OH	H	fisetin
		OH	=O	OH	H	OH	H	H	OH	OH	H	quercetin
		OH	=O	OH	H	OH	H	H	OH	OH	H	myricetin
Flavan												
		H	=O	H	H	H	H	H	H	H	H	flavanone
		H	=O	OH	H	OH	H	H	H	OH	H	4′.5.7-trihydroxy flavanone
		OH	H	OH	H	OH	H	H	OH	OH	H	(±)-catechin
		OH	H	OH	H	OH	H	H	OH	OH	H	(−)-epicatechin
Coumarin												
	=O	H	H	H	H	H	H					coumarin
	=O	APE[b]	OH	H	H	H	H					warfarin
	=O	H	H	H	Ome	OMe	H					6,7-dimethoxy-coumarin
	=O	H	Ph	H	H	OH	H					7-hydroxy-4-phenylcoumarin
	=O	H	Ph	H	H	OMe	H					7-methyl-4-phenyldihydrocoumarin
	=O	APE[b]	OH	H	H	H	H					3-(2-acetyl-1-phenylethyl)-4-hydroxy-coumarin
Stibenes												diethylstilbestrol
Saponins												18α-glycyrrhetinic acid
Plant sterols												β-sitosterol
Fungi resorcylic acid lactones												zearalenone

[a] 3β-HSD activities were assayed in duplicates at 25°C in a standard 1 ml pH 7.5 assay medium containing 100 mM KPi, 20% glycerol, 0.1 mM EDTA, 2% MeOH, 2μM pregnenolone, 0.8 mM NAD^+, 0.1 nM enzyme and various concentrations of inhibitor. No inhibition up to 10 μM.
[b] APE: 2-Acetyl-1-phenylethyl.

Table 6
IC_{50} values for daidzein, genistein, biochanin A and formononetin inhibition of bovine adrenal 3β-HSD toward different hydroxysteroid substrates[a]

3β-hydroxysteroids	IC_{50} (μM)			
	formononetin	daidzein	genistein	biochanin A
Pregnenolone	3.7	1.5	1.5	3.7
Dehydroepiandrosterone	3.5	11.0	10.0	7.5
5α-pregnan-3β-ol-20-one	0.4	1.3	0.9	0.5
Androstenediol	5.8	4.0	4.0	ND

[a] 3β-HSD activities were assayed in triplicates at 25°C in a standard 1 ml pH 7.5 assay medium containing 100 mM KPi, 20% glycerol, 0.1 mM EDTA, 2% MeOH, 0.8 mM NAD^+, 0.1 nM enzyme and various concentrations of inhibitor. Steroid substrates concentration is equal to K_m value. IC_{50} values are inhibitor concentrations at which enzyme reaction rates were suppressed by 50%. ND = not determined.

The IC_{50} values of the isoflavone inhibitors were determined at substrate concentrations equal to K_m. Hence, these values should be very similar to their corresponding inhibition constants (K_i values). Although the sites and mechanisms of action of the isoflavone inhibitors are unknown at this time, the facts that they are structurally similar to the hydroxysteroid substrates and that they only inhibit γ-type ADH suggest that they may act at the hydroxysteroid binding site of these enzymes. The fact that bacterial β-HSD is more susceptible to isoflavone inhibition (IC_{50} in nM range) [28] supports the notion that the active site of the bacterial enzyme is more flexible and can better accommodate structurally diversed substrates and inhibitors.

Thomas et al. postulated that the human placental 3β-HSD/isomerase is a single enzyme with dual, dehydrogenase and isomerase, activities. It is believed that the oxidation and isomerization reactions are carried out at the same 'active site' on the enzyme [2]. The site(s) of action of the bovine adrenal 3β-HSD/isomerase have not been clarified. In the present study, we have shown that: (i) the IC_{50} values of the isoflavone inhibitors measured for the 3β-HSD activity using pregnenolone as the substrate are very similar to those measured for the isomerase activity using Δ^5-pregnen-3,20-dione, the intermediate of the 3β-HSD reaction, as the substrate (Table 4), (ii) the 3β-HSD and isomerase activity were co-purified from bovine adrenal and (iii) the purified enzyme is homogeneous on SDS gel. Based on these results, we suggest that like the human placental enzyme, bovine adrenal 3β-HSD/isomerase is a single protein capable of catalyzing both the 3β-HSD and isomerase reactions, presumably on the same 'active site.' However, further studies, e.g. modification of the active sites in the enzyme are required to confirm this hypothesis.

Table 7
Effect of isoflavones, flavone, flavan, coumarin and phytoestrogens on the isomerase activity of bovine adrenal 3β-HSD/isomerase[a]

Type	Name	IC_{50} (μM)
Isoflavones	daidzein	3.8
	biochanin A	0.4
	genistein	2.3
	formononetin	4.0
	daidzin	NI
	genistin	NI
	puerarin	NI
	prunetin	NI
Estrogen	17β-estradiol	0.3
Saponins	18α-glycyrrhetinic acid	NI
Plant sterols	β-sitosterol	NI
Fungi resorcylic acid lactones	zearalenone	NI
Flavone	flavone	NI
	apigenin	NI
Flavan	flavanone	NI
Coumarin	coumarin	NI
Stibenes	diethylstilbestrol	NI

[a] Isomerase activities were assayed in triplicates at 25°C in a standard 1 ml pH 7.5 assay medium containing 100 mM KPi, 20% glycerol, 0.1 mM EDTA, 2% MeOH, 0.1 mM NAD^+, 0.025 nM enzyme, 10 μM Δ^5-pregnen 3,20-dione and various concentration of inhibitors. NI = no inhibition up to 10 μM.

Although all isoflavones that inhibit 3β-HSD/isomerase are also phytoestrogens, not all phytoestrogens inhibit 3β-HSD/isomerase, for instance, 18α-glycyrrhetinic acid, β-sitosterol, zearalenone. Therefore, it is rather unlikely that these isoflavones exert their estrogenic/anti-estrogenic activities by inhibiting of 3β-HSD/isomerase. Further, unlike aromatase, 3β-HSD/isomerase is not a key enzyme of and does not play a key role in the biosynthesis of estrogens. It is generally believed that the estrogenic/anti-estrogenic activity of isoflavones is mediated by binding to the estrogen receptor in target tissues [15,40].

The structural resemblance of the isoflavone inhibitors e.g. daidzein and genistein, to the 3β-hydroxysteroid substrates, e.g. pregnenolone and DHEA is believed to account for their inhibitory action on 3β-HSD/isomerase. Survey of a series of isoflavones and related compounds revealed some structure-function relationship. First, the 1,2-diphenylpropane skeleton appears to be essential for 3β-HSD inhibition because among the compounds tested, only isoflavones but not

flavones inhibit the enzyme (Table 4,5). A free 7-hydroxy group also appears to be important. Hence, analogs with a blocked 7-hydroxy group such as prunetin, the 7-O-Me-derivative of genistein and daidzin, the 7-O-glucosyl-derivative of daidzein, do not inhibit 3β-HSD/isomerase (Table 5). An additional hydroxyl group at the 5 position (daidzein vs. genistein) and a blocked 4′-hydroxy group, formononetin and biochanin A vs. daidzein and genistein, respectively, do not appear to be critical for inhibition (Table 4).

The fact that daidzein and other related isoflavones potently inhibit bovine adrenal 3β-HSD activity (the present study) and other steroid metabolizing enzymes e.g. human aromatase/estrogen synthetase [11] and 17β-hydroxysteroid oxidoreductase [26], suggests that they might exert some of their biological activities via one or more steroidal pathways. Recently, steroids have attracted particular attention in behavioral sciences because some of them have been shown to play important roles in the regulation of the behavioral and psychophysiological phenomena, e.g. aggression, appetite, stress, seizures, sleep, anxiety, stress, memory, sexual functions, premenstrual syndrome and postpartum depression etc [41]. These steroids, now known as neurosteroids, exert their effects by acting either as allosteric agonists or antagonists of neuroral receptors such as the amino acid neurotransmitter GABA-, NMDA-, glycine- and glutamate- receptors. Pregnenolone sulfate (PS), the sulfate derivative of pregnenolone, is a noncompetitive antagonist of the $GABA_A$ receptor [42] and glycine receptor [43], the two major inhibitory neurotransmitters in CNS mediating fast synaptic inhibition via the activation of the receptor-linked chloride ionophore [44]. DHEA [45] and its sulfate [42] also act as allosteric antagonists of $GABA_A$ receptor. On the other hand, neurosteroids, such as androsterone [46], progesterone [43], the reduced A-ring metabolites of progesterone: 5α-pregnan-3β-ol-20-one (allopregnanolone) [47]; 3α-pregnan-5α-21-diol-20-one (3α-THDOC) [48]; tetrahydroprogesterone (THP; 5α-pregnan-3α-ol-20-one) [42] and allotetrahydrodeoxycorticosterone/allotetrahydroDOC (THDOC, 5α-pregnan-3α,21-diol-20-one) [42] are agonists of $GABA_A$ receptor. These GABAergic steroids stimulate Cl^- uptake into rat brain synaptoneurosomes and enhance GABA-activated Cl^- conductance in hippocampal neurones [49]. Furthermore, pregnenolone sulfate has been shown to act as a positive allosteric modulator of the *N*-methyl-D-aspartate (NMDA) receptor [50] and to enhance the response of the glutamate receptor [30]. DHEA sulfate and progesterone is an agonist and an antagonist to the NMDA receptor, respectively [51]. In contrast to GABA and glycine, NMDA and glutamate belong to the excitatory neurotransmitter.

Neurosteroids such as DHEA, DHEA sulfate, THP, pregnenolone and pregnenolone sulfate can be synthesized and accumulated in the brain and are not solely dependent on the supply of peripheral endocrine glands. In fact, pregnenolone, DHEA and their sulfate derivatives are synthesized and accumulated in mammalian brains [52] at concentrations largely exceed those in plasma [53]. The absolute and relative concentrations of these neurosteroids are controlled by the activities of the steroid metabolizing enzymes involved. Among these, 3β-HSD/isomerase, steroid sulfatase, hydroxysteroid sulfotransferase, 5α-steroid reductase and 3α-hydroxysteroid oxidoreductase appear to be directly involved in the metabolism and distribution of DHEA ($GABA_A$ antagonist), progesterone (NMDA, glycine antagoinst), DHEA sulfate and pregnenolone sulfate ($GABA_A$ antagonist, NMDA agonist), allopregnanolone and allotetrahydrodoc ($GABA_A$ agonist) in brain. The 3β-HSD/isomerase, the key enzyme in the biosynthesis of neurosteroids in brain, is expressed in several particular brain areas [9]. Isoflavones may affect these neurosteroid systems by inhibiting 3β-HSD/isomerase.

In the present study, IC_{50} values determined for the isoflavone inhibitors range from 0.4 to 11 μM, within the concentration ranges of daidzein and genistein found in the plasma (0.4 μM) [54] and urine (12 μmol per 24 h) [55] of individuals on vegetarian or semi-vegetarian diet. The exact concentrations of isoflavones in human liver, adrenals and other tissues are unknown at this time. However, in animal experiments, we have shown that the isoflavone daidzin administered subchronically accumulates in liver (the target tissue of daidzin). Liver daidzin concentrations could reach 70 μM, more than 10 times higher than the maximal plasma concentration obtained in pharmacokinetic studies [56,57]. Therefore, it is very likely that these isoflavones will also accumulate in their target tissues (e.g. adrenal, liver etc.) and reach concentrations that are well above IC_{50} values reported in this study. Hence, dietary isoflavones may exert some of their biological effects by inhibiting the action of 3β-HSD/isomerase, a key enzyme of neurosteroid and/or other steroid hormone biosynthesis.

The isoflavone daidzein does not affect the activities of partially purified hamster cytosolic hydroxysteroid sulfotransferase and the microsomal steroid sulfatase preparations (Table 1). However, it has been shown that the sulfonconjugates of daidzein are potent inhibitors of sterol sulfatase [58]. Hence, daidzein and presumably other isoflavones as well, could modulate steroid sulfoconjugation and hydrolysis via their secondary metabolites such as daidzein-sulfoconjugates.

It has recently been shown that DHEA reduces some aging associated deficits in gene level [59,60] and activated the immune function in animal model [61,62]. Therefore, inhibition of 3β-HSD may indirectly elevate the level of DHEA synthesis to reverse the influence of the aging process. Other studies have associ-

ated high dietary isoflavones intake with low incidence of breast cancer. As mentioned above, dietary isoflavones such as daidzein undergoes sulfoconjugation and forms both daidzein-4′-*O*-sulfate and daidzein-7,4′-di-*O*-sulfate [63]. We have shown that daidzein sulfoconjugates are potent inhibitors of sterol sulfatase, a key enzyme involved in the conversion of inactive forms of estrogens (sulfoconjugates of estrone androstenediol) into active forms estrogens (estrone, estradiol and androstenediol). This process is believed to play an important role in the genesis of breast cancer, especially in postmenopausal women [58].

Isoflavones have also been shown to inhibit 17β-hydroxysteroid oxidoreductase for the 17β-oxidoreduction of estrogens in breast and prostate cancer cells [64]. The consumption of isoflavones was also found to reduce serum estrogen concentration in premenopausal Japanese women [65]. Our previous studies showed that isoflavones may reduce the synthesis of estradiol by influencing the synthetic pathway of estradiol. These findings, together with the data reported here suggest that dietary isoflavones may act in a concerted way on steroid metabolizing enzymes and provide the biochemical basis for the vast beneficial effects of vegetarian and semi-vegetarian diets.

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