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Soy isoflavones interfere with thyroid hormone homeostasis in orchidectomized middle-aged rats

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ABSTRACT

We previously reported that genistein (G) and daidzein (D) administered subcutaneously (10 mg/kg) induce changes in the angio-follicular units of the thyroid gland, reduce concentration of total thyroid hormones (TH) and increase thyrotropin (TSH) in serum of orchidectomized middle-aged (16-month-old) rats. To further investigate these effects, we now examined expression levels of the thyroglobulin (*Tg*), thyroperoxidase (*Tpo*), vascular endothelial growth factor A (*Vegfa*) and deiodinase type 1 (*Dio 1*) genes in the thyroid; in the pituitary, genes involved in TH feedback control (*Tsh β*, *Dio 1*, *Dio 2*, *Trh* receptor); and in the liver and kidney, expression of T_3-activated genes *Dio 1* and *Spot 14*, as well as transthyretin (*Ttr*), by quantitative real-time PCR. We also analyzed TPO-immunopositivity and immunofluorescence of T_4 bound to Tg, determined thyroid T_4 levels and measured deiodinase enzyme activities in examined organs. Decreased expression of *Tg* and *Tpo* genes ($p < 0.05$) correlated with immunohistochemical staining results, and together with decreased serum total T_4 levels, indicates decreased Tg and TH synthesis following treatments with both isoflavones. However, expression of *Spot 14* ($p < 0.05$) gene in liver and kidney was up-regulated, and liver *Dio 1* expression and activity ($p < 0.05$) increased. At the level of pituitary, no significant change in gene expression levels, or Dio 1 and 2 enzyme activities was observed. In conclusion, both G and D impaired Tg and TH synthesis, but at the same time increased tissue availability of TH in peripheral tissues of Orx middle-aged rats.

Introduction

Soybean diet was first described to exert goitrogenic effects in domestic animals (McCarrison, 1993). Later on, goiter and hypothyroidism were reported in infants fed with adapted soy formula without adequate iodine supply (Van Wyk et al., 1959). This adverse effect was eliminated by supplementing commercial soy infant formulas with iodine, or by switching to cow milk (Chorazy et al., 1995). Results of clinical studies with healthy individuals were inconsistent, reporting that isoflavones have significant, mild or no effect on thyroid function (Bitto et al., 2010). However, in a recent randomized double-blind crossover study with subclinically hypothyroid patients receiving soy-based phytoestrogens containing G and D for 8 weeks, six of sixty patients developed overt hypothyroidism indicating interference of soy isoflavones with the thyroid hormone axis (Sathyapalan et al., 2011).

In animal models, rodents in particular, several authors reported induction of goiter in rats fed a soybean diet, however only in cases of iodine deficiency or presence of some other goitrogenic factors (Ikeda et al., 2000; Kajiya et al., 2005; Kimura et al., 1976).

Doerge and his associates identified the direct molecular target of isoflavone action in the thyroid tissue: both genistein (G), and to a lesser extent daidzein (D) were shown to strongly inhibit the activity of thyroid peroxidase (TPO), the key enzyme in the synthesis of thyroid hormones (TH), both in vitro and in vivo (Chang and Doerge, 2000; Divi et al., 1997; Doerge and Sheehan, 2002).

In addition, G, and to a lesser extent D, were reported to be potent competitors of T_4 for binding to transthyretin (TTR), the serum and cerebrospinal fluid (CSF) thyroid hormone distributor protein, in vitro (Radović et al., 2006). TTR is the major plasma carrier of thyroid hormones in rodents (Davis et al., 1970) and is the main TH-binding protein in CSF of both rodent and humans (Hagen and Solberg, 1974). Displacement of TH from TTR may increase free thyroid hormone levels and subsequently alter the thyroid hormone homeostasis, such as enhanced tissue availability of TH and increased urinary elimination (Köhrle

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et al., 1989). However, all researchers who examined the effects of isoflavone treatments on serum thyroid hormone levels in young adult rodents obtained no significant change when iodine was provided at sufficient levels (Chang and Doerge, 2000; Gotthardt, 2009; Schmutzler et al., 2004; Šošić-Jurjević et al., 2013).

Aging is associated with alterations in the function of the hypothalamus–pituitary–thyroid axis. Serum thyrotropin-releasing hormone (TRH) levels decrease in aged rat hypothalamus (Cizza et al., 1992), while normal circulating levels of thyrotropin (TSH) were reported, despite low serum thyroxine (T_4) levels (Donda and Lemarchand-Béraud, 1989; Moreira et al., 2005; Reymond et al., 1992; Sosic-Jurjevic et al., 2012). Besides the hormonal changes, in line with other researchers (Mariotti et al., 1995; Reymond et al., 1992), we detected presence of inactive follicles in thyroid tissue of middle-aged rats (Sosic-Jurjevic et al., 2012; Sosić-Jurjević et al., 2005). These follicles were large in size and filled with dense colloid.

Encouraged by aggressive advertisement, the elderly population tends to increasingly use isoflavones as nutritional supplements, despite the fact that the prevalence of thyroid dysfunction increases with age (Eisenbrand, 2007). In addition, a significant percentage of elderly people and centenarians have elevated serum TSH concentrations (Atzmon et al., 2009), which is in some studies associated with a decline in serum free T_4 levels (Surks and Hollowell, 2007). More recent data indicate that beneficial effects of soy supplements in menopausal women are minimal, and pose a risk to breast cancer patients (Messina, 2010). However, by preventing prostate cancer development, soy isoflavones may actually have more impact on health of the male population (Wuttke et al., 2010). The results on isoflavone effects in aged humans and rodents are scarce.

We previously demonstrated for the first time that administrating G or D to orchidectomized (Orx) middle-aged rats, fed a soy-free diet with sufficient iodine content induce micro-follicular changes in the thyroid tissue (including hypertrophy of Tg-immunopositive follicular epithelium and colloid depletion), accompanied by reduced serum level of thyroid hormones, and higher TSH. The effect on TSH was more noticeable in case of daidzein (Sosić-Jurjević et al., 2010). More in depth analysis of Orx middle-aged model revealed decreased liver Dio1 and pituitary Dio2 enzyme activities, despite unchanged serum total T_4 and TSH, in comparison to age-matched sham-operated controls (Sosic-Jurjevic et al., 2012).

Based on the previous results, we hypothesized that administration of soy isoflavones to Orx middle-aged rats has primarily suppressed thyroid function, which consequently resulted in elevated TSH levels in serum. In the present study we aimed to test this hypothesis and to further examine how G and D interfered with thyroid homeostasis in this animal model. Therefore, we determined expression levels of most relevant genes in the major tissues controlling the HPT axis, the thyroid, liver, kidney, and pituitary. We also performed the additional morpho-functional characterization of thyroid tissue and Dio enzyme activities in the corresponding organs as sensitive endpoints of TH action.

Materials and methods

Animals and diets. Male Wistar rats were housed in the unit for experimental animals at the Institute for biological research "Siniša Stanković". They were kept individually under constant conditions: 12-h light/12-h dark cycle and constant temperature (22 ± 2 °C).

Two weeks prior to the experiments, the animals were put on a soy-free diet. This diet was prepared according to Picherit et al. (2000) in cooperation with the Department of Food, School of Veterinary Medicine, Belgrade, Serbia. The food contained (per 100 g): casein, 20.3; cornstarch, 45; sucrose, 20; corn oil, 5.2; fiber, 3.7; vitamin/mineral mix (Ca-P Deficient), 1.5; calcium phosphate dibasic, 1.8; calcium carbonate, 1 g; sodium chloride (iodized with 38.8 ± 6.5 mg of potassium iodide per kg of salt), 0.5. Casein and crystalline cellulose were purchased from Alfa Aesar, Johnson Matthey GmbH & Co. KG, Karlsruhe, Germany. Salt was obtained from Salinen d.o.o., Šimanovci, Serbia. All other ingredients were from Fish Corp. 2000, Belgrade–Subotica, Serbia.

Experimental protocol. At the age of 15–16 months, animals were bilaterally orchidectomized (Orx; n = 34) under ketamine anesthesia (15 mg/kg b.w.; ketamidor 10%, Richter Pharma, Wels, Austria).

Two weeks after the surgery, rats were divided into groups according to the treatments they received. Ten milligrams per kilogram b.w. of genistein or daidzein (LC laboratories, MA, USA) were injected subcutaneously (*s.c.*) to orchidectomized Orx + G (n = 12) and Orx + D (n = 12) rats, respectively. They were dissolved in a minimal volume of absolute ethanol and mixed with sterile olive oil (ratio 1:9). The substances were administered daily for three weeks. Rats in the Orx (n = 12) group were administered *s.c.* with the same volume of vehicle solution according to the same schedule.

The animals were decapitated 24 h after the last treatment. Urine was collected before killing. Blood was collected from the trunk. The sera and urine samples were stored at −80 °C until assayed. Pituitaries, livers and kidneys were removed, immediately frozen in liquid nitrogen and stored at −80 °C for further processing. Thyroid lobes from each animal were separated, and then randomly chosen to be further processed either for histological examination (n = 5), or frozen in liquid nitrogen and stored at −80 °C (n = 19). The criteria for choosing number of animals for each analysis (and determination of each endpoint) were to reduce animal use while increasing the scientific validity of the results. The organs from approximately six randomly chosen animals were used for molecular analyses (n = 5–6 in case of pituitary, and n = 6–8 in case of thyroid, liver or kidney), while organs from another group of five to six randomly chosen animals were used for histological examinations, Dio assays, or for determination of tissue hormone concentration.

This study was approved by the Animal Care and Use Committee of our Institute, following recommendations provided in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123, Appendix A).

Measurement of isoflavone levels in sera and urine. Serum (n = 8) and urine samples (n = 8) were dried by Freeze Dryers Rotational-Vacuum-Concentrator, GAMMA 1-16 LSC, Germany, and sample portions of dry weight were used for measuring of serum G and D levels by the gas chromatography–mass spectrometry (GC–MS) and quantitative nuclear magnetic resonance (qNMR) methods as detailed bellow.

For GC–MS analysis, a portion of lyophilized urine (10 μl/ml of the original urine) was adjusted to pH 5.2 with acetic acid (1 mol/l) and incubated at 50 °C for 1.5 h with a mixture of β-glucuronidase from *Escherichia coli* (10–20 U per μL of urine; Sigma-Aldrich, Saint Louis, MO, USA), then acidified with 1/30 volume of 12 mol/l HCl and treated by adding 2.8 volumes of acetone. The resulting mixtures were centrifuged for 5 min at 12,000 ×*g* at room temperature. Supernatants were carefully evaporated to dryness at 56 °C under a stream of nitrogen. The residue was redissolved in 50 μl of derivatization reagent (*N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide with 1% *tert*-butylchlorodimethylsilane; Sigma-Aldrich, Saint Louis, MO, USA) and sonicated for 1 min. Sample was kept at 75 °C for 30 min under shaking at 234 ×*g*. After cooling, the surplus reagent was removed at room temperature under a stream of nitrogen. The dried residues were dissolved in a known volume of hexane, sonicated for 1 min, centrifuged (12,000 ×*g*, 1 min) and transferred to autosampler vials. As a negative control, distilled water was treated in the same way.

The GC–MS analyses were performed on a Hewlett-Packard 6890N gas chromatograph equipped with a fused silica capillary column DB-5MS (5% phenylmethylsiloxane, 30 m × 0.25 mm, film thickness