Goitrogenic and Estrogenic Activity of Soy Isoflavones

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Soy is known to produce estrogenic isoflavones. Here, we briefly review the evidence for binding of isoflavones to the estrogen receptor, *in vivo* estrogenicity and developmental toxicity, and estrogen developmental carcinogenesis in rats. Genistein, the major soy isoflavone, also has a frank estrogenic effect in women. We then focus on evidence from animal and human studies suggesting a link between soy consumption and goiter, an activity independent of estrogenicity. Iodine deficiency greatly increases soy antithyroid effects, whereas iodine supplementation is protective. Thus, soy effects on the thyroid involve the critical relationship between iodine status and thyroid function. In rats consuming genistein-fortified diets, genistein was measured in the thyroid at levels that produced dose-dependent and significant inactivation of rat and human thyroid peroxidase (TPO) *in vitro*. Furthermore, rat TPO activity was dose-dependently reduced by up to 80%. Although these effects are clear and reproducible, other measures of thyroid function *in vivo* (serum levels of triiodothyronine, thyroxine, and thyroid-stimulating hormone; thyroid weight; and thyroid histopathology) were all normal. Additional factors appear necessary for soy to cause overt thyroid toxicity. These clearly include iodine deficiency but may also include additional soy components, other defects of hormone synthesis, or additional goitrogenic dietary factors. Although safety testing of natural products, including soy products, is not required, the possibility that widely consumed soy products may cause harm in the human population via either or both estrogenic and goitrogenic activities is of concern. Rigorous, high-quality experimental and human research into soy toxicity is the best way to address these concerns. Similar studies in wildlife populations are also appropriate. *Key words:* estrogen toxicity, estrogenicity, genistein, isoflavones, mass spectrometry, soy, thyroid peroxidase, thyroid toxicity. *Environ Health Perspect* 110(suppl 3):349–353 (2002).
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The potential health benefits of soy, and the soy isoflavones in particular, are widely publicized. Although soy and soy isoflavones exhibit both risks and benefits (*1,2*), in this article we focus on the thyroid toxicity of genistein. Genistein is the major isoflavone synthesized by the soybean; genistein possesses both estrogenic and goitrogenic activities. The claimed benefits of genistein are being examined in numerous experimental, epidemiologic, and clinical studies investigating breast and prostate cancer chemoprevention, relief of postmenopausal symptoms, and prevention or slowing of osteoporosis. Here, we first present a brief summary of the estrogenic activity and toxicity of genistein and then explore the potential for thyroid toxicity of these chemicals, both from a historical perspective and from data reported from recent investigations on the mechanisms of potential toxicity. We also integrate research results on isoflavone thyroid effects in a manner useful for predicting and identifying potential risks from soy consumption in various human populations.

Soy and Isoflavone Estrogenic Activity

Phytoestrogens comprise a class of several different chemicals produced by a variety of plants (*2*). Of these, the soy isoflavones (particularly genistein) are of greatest interest because of the widespread human consumption of soy, due largely in Western countries to extensive advertising by the soy industry for potential human health benefits. However, despite the widespread belief that soy consumption is safe, soy isoflavones administered during development can cause several forms of estrogen toxicity in experimental animals.

As part of a large project to develop a battery of predictive computational models, we recently assayed 230 chemicals for binding to the estrogen receptor (ER) (*3*). Of these, 46 were phytoestrogens from six different chemical structure classes. Of the nine isoflavones, seven bound the ER with measurable affinity, ranging from a relative binding affinity (RBA) of 0.45 for genistein to 0.0013 for formononetin, with the RBA for estradiol being 100. Equol, a metabolite of the phytoestrogen daidzein, had an RBA for ER that was 33% that of genistein (*4*). When examined in a battery of rat *in vivo* assays developed to assess estrogen activity and toxicity during postnatal development, equol increased uterine weight. Also, equol treatment on postnatal days (PNDs) 1–5 inhibited uterine weight gain on PNDs 20 and 25, as did coumestrol and diethylstilbestrol (DES) (*4*). After PND 10–14 treatment, equol, like coumestrol and DES, inhibited the development of uterine glands. This was a frank toxic response because the entire uterine tissue compartment was mostly absent. Recently, genistein was found to cause uterine adenocarcinoma in adult mice, following neonatal treatment (*5*). Because earlier DES studies showed the same effect, equipotent doses of genistein and DES (the positive control) were chosen based on uterotrophic activity. The tumor incidence was statistically the same in both the DES and genistein groups. This finding strongly suggests that the estrogenic activity per se, and not the chemical structure, is responsible for this malignant outcome.

When assessed in women in a controlled trial, a dose of 30 g soy flour/day had frank estrogenic effects, including lengthening of the menstrual cycle (*6*). Infants on soy infant formula receive a dose of phytoestrogens that is 5-fold higher than the dose causing estrogenic effects in women (*7*). This level of soy isoflavone exposure to approximately 20% of American infants should be of concern, but no robust studies in infants have been conducted.

The evidence outlined here is sufficient to conclude that genistein and equol bind to the ER, are estrogenic *in vivo*, and are estrogenic developmental toxicants; that genistein is an estrogenic carcinogen in rodents; and that such exposures may be relevant to humans.

Goitrogenic Activity of Soy Isoflavones

Literature Review

It is well described but little known that the soybean and goiter have long been associated in animals and humans. Rodents are useful risk assessment models for thyroid toxicants, despite significant differences between rodent

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and human thyroid physiology (*8*). In rats the goitrogenic activity of soy and its inhibition by dietary iodide supplementation have been defined (*9–13*). The negative interaction of low dietary iodine and soy is demonstrated by the finding of Kimura et al. that thyroid carcinoma appeared in rats fed an iodine-deficient diet consisting of 30% defatted soy (*14*). In humans, goiter has been seen in infants fed soy formula; this is usually reversed by changing to cow milk or iodine-supplemented diets (*15–22*). After the 1960s, manufacturers reportedly began adding iodine to formulas to mitigate thyroid effects. Fort et al. (*23*) conducted a retrospective epidemiologic study on teenage children diagnosed with autoimmune thyroid diseases (Hashimoto's thyroiditis or Graves' disease). Those consuming soy formula as infants had twice the prevalence of autoimmune disease (18 of 59, 31%) of healthy siblings (9 of 76, 12%) or controls (7 of 54, 13%). Goiter and high normal thyroid-stimulating hormone (TSH) levels in healthy iodine-sufficient adults occurred as early as 1 month (n = 37) after commencing a diet that included 30 g of pickled soybeans per day (*24*). Although it was not measured, dietary iodine content may have been insufficient to protect against the antithyroid effect of soy. Furthermore, no changes in serum thyroid hormone [triiodothyronine (T_3) and thyroxine (T_4)] levels were found. After 1 month off the soy diet, TSH decreased to the pretreatment levels and goiters were diminished in size. Lowered T_3 levels were seen in 14 premenopausal but not in 18 postmenopausal women on a soy diet (up to 2 mg total of soy isoflavones per kilogram body weight per day) for about 3 months (*25*). Interestingly, in another study, as little as 1 month of soy supplementation decreased T_3/T_4 levels during the luteal phase of the menstrual cycle, but levels increased during the follicular phase (*26*).

Biosynthesis of Thyroid Hormones and Inhibition by Antithyroid Chemicals

Thyroid peroxidase (TPO) is found in the apical membrane of thyroid follicular cells. TPO, a heme-containing enzyme, catalyzes both reactions required for thyroid hormone synthesis (see Scheme 1). The first step is iodination of thyroglobulin tyrosyl residues, followed by oxidative coupling to yield T_4 and T_3. Inhibition of porcine TPO activity is a mechanism common to many classes of synthetic antithyroid compounds (*27–31*) and naturally occurring flavonoids (*32,33*). Lactoperoxidase (LPO) is often used as a model for TPO, based on many shared structural and functional properties. For this reason investigations were started on soy isoflavone inhibition of both porcine TPO and LPO activity. Genistein and daidzein were found to be the chemicals in soy that inhibited both TPO-catalyzed iodination and coupling (*33,34*). The nature of the inhibition of enzymatic activity under various conditions is quite interesting, if not startling. First, absent iodide, genistein and daidzein act as suicide substrates for TPO and LPO by covalently binding to the active site. This was shown by the irreversible loss of both iodinating and coupling activities and concomitant changes in the ultraviolet-visible spectrum of the enzyme. Second, with adequate iodide, genistein and daidzein are alternate substrates; products are mono-, di-, and triiodoisoflavones.

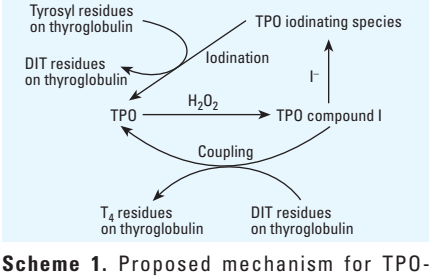
Scheme 2 proposes mechanisms by which genistein can intercept reactive enzyme intermediates involved in the iodination and coupling reactions required for T_4 synthesis. Mechanisms include reaction of compound I with isoflavones that could produce a reactive isoflavone radical at the active site, along with a radical form of compound II, which could combine to form inactivated enzyme presumably through covalent modification of active site amino acid residues. Consistent with this hypothesis are the covalent binding of approximately 3 mol of radiolabeled genistein per 1 mol of inactivated LPO; the unchanged heme content in inactivated LPO (data not shown); and the ultraviolet-visible spectral changes observed upon inactivation of LPO and TPO (*33*).

Inactivation of TPO by Isoflavones *in Vitro*

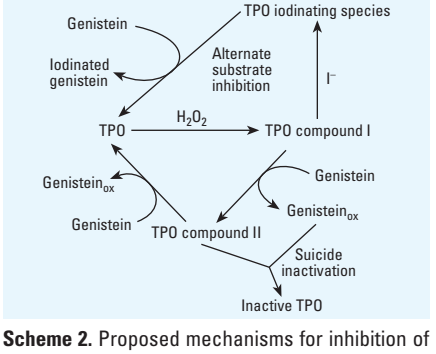
Microsomal rat TPO (rTPO; from untreated animals) incubated with genistein and hydrogen peroxide (H_2O_2) was used to characterize isoflavone-mediated, time-dependent TPO inactivation *in vitro* (*35*) (Figure 1). The control experiments demonstrate that neither H_2O_2 nor genistein alone altered activity, consistent with the suicide inactivation mechanism previously proposed (*33,35*) and Scheme 2. The apparent inhibition binding constant, K_i, and the maximal inactivation rate constant, k_{inact}, were 50 nM and 0.28 min^{-1}, respectively. Daidzein likewise inactivated TPO, with constants of 143 nM and 0.31 min^{-1}. These kinetic parameters are consistent with very potent inactivation, unlike other dietary flavonoids tested (*32*), and suggest a mechanism for low-dose effects of soy. The sensitivity of microsomal rTPO to inactivation by genistein was compared with other mammalian peroxidases (*35*). Purified bovine LPO, porcine TPO, human TPO, and microsomal rTPO all showed 40–66% inactivation at 30 min, suggesting that isoflavone-mediated inactivation of TPO is a general phenomenon across mammalian species.

Dietary Exposure of Sprague-Dawley Rats to Genistein

The observed isoflavone inhibition of peroxidase activity *in vitro* led to treatment of Sprague-Dawley rats with genistein to investigate possible endocrine disruption in both dose-range-finding (*36*) and multiple-generation studies (in progress). Genistein doses of 0, 5, 100, and 500 ppm were administered in soy-free basal diet (total genistein and daidzein ~0.5 ppm each) to pregnant female rats 4 weeks before mating through pup weaning at PND 21. This was followed



Scheme 1. Proposed mechanism for TPO-catalyzed T_4 synthesis. DIT, diiodotyrosine.



Scheme 2. Proposed mechanisms for inhibition of TPO by soy isoflavones.

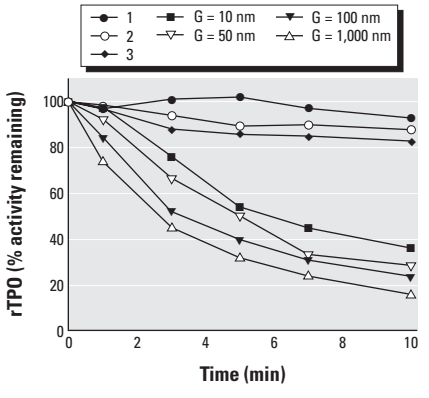


Figure 1. Inactivation of rat TPO by genistein *in vitro*. Microsomal rTPO was incubated with the indicated concentrations of genistein in the presence of H_2O_2 (100 nM) at room temperature in 0.1 M phosphate buffer, pH 7.0. At various times, aliquots were removed and remaining activity determined using a spectrophotometric guaiacol oxidation assay. 1: control, rTPO alone; 2: control, rTPO + H_2O_2; 3: control, rTPO + 1,000 nM genistein. G = concentration of genistein added to TPO and H_2O_2. Data from Chang and Doerge (*35*).

Table 1. Genistein consumption and serum levels in Sprague-Dawley rats.

Genistein dose (μg/g in diet)[a]	Estimated genistein intake (mg/kg/day)[b]	Concentration total genistein (μM)[c]	
		Males	Females
Basal	0.04	<0.01	<0.01
5	0.4	0.06 ± 0.006	0.10 ± 0.008
100	8	0.59 ± 0.030	0.94 ± 0.21
500	40	6.00 ± 0.65	7.94 ± 2.47

[a]The basal soy-free diet, which contained approximately 0.5 ppm total genistein and daidzein, was fortified genistein aglycone at the indicated level. [b]Feed consumption and body weights were measured for individual animals. [c]Total genistein concentration was measured in serum from male and female rats using LC-ES/MS (limit of quantitation, 0.01 μM).

Table 2. Isoflavone[a] intake and blood concentrations in humans.

Population group—soy form	Total isoflavones (mg/kg/day)[b]	Blood isoflavones (μM)
Adults—Western diet	Very low	Very low
Adults—Asian diet	<1[c]	0.1–1.2[d]
Adults—soy nutritional supplement	0.7[e]	0.5–0.9[e]
Infants—soy formula	6–9[f]	2–7[f]
Adults—soy cancer supplement	200[g]	?

[a]Total active isoflavones = genistein + daidzein. [b]70 kg body weight. [c]Soy isoflavone intake from typical Japanese diet estimated at 30 mg/day (*26*). [d]From six Japanese men (*37*). [e]From three adult volunteers (*38*). [f]From seven infants (*6*). [g]From label instructions.

by consumption of the same diet until offspring were sacrificed at PND 140. Total blood genistein (i.e., both conjugates and aglycone) was measured using electrospray mass spectrometry (ES/MS) (Table 1) (*37*). The range of total genistein traversed the range found in several human populations (Table 2) (*38,39*). On the 500 ppm diet, rat genistein levels were similar to those in infants on soy formulas (*7*). Rats on the 100 ppm diet had genistein levels similar to those found in adults on typical Asian diets (*38*) or soy isoflavone dietary supplements (*39*). Rats consuming 5 ppm and control diets had low genistein levels typically found with a Western diet (Table 1). Female rats had higher blood genistein levels than males, consistent with the sex-specific difference in genistein elimination half-time (3.0 vs. 4.3 hr in males and females, respectively) (*37*). The predominant circulating metabolites (97–99%) are genistein glucuronides in both rats (*40*) and humans (*39*). The predominance of circulating metabolites (97–99%) is consistent with extensive first-pass metabolism of genistein in the gut and/or liver (*41*).

Intrathyroidal Accumulation of Genistein

Both total and aglycone genistein concentrations were measured in thyroids using liquid chromatography (LC)-ES/MS (*35*). Figure 2 shows the results for females and males; the higher average thyroidal levels observed in females reflected the higher average blood concentrations. Thyroid genistein aglycone (i.e., the unconjugated form) was substantially increased relative to that in blood (18–28% vs. 1–3%). Total thyroidal genistein occurs in the range of 0.1–1.2 nmol/g tissues and for the aglycone, 0.1–0.3 nmol/g. Because water accounts for slightly less than two-thirds of thyroid weight, concentrations of genistein aglycone were up to 350 nM. Elevated tissue aglycone levels, up to 100% of total, were also measured in several male and female reproductive organs (*37*). This demonstrates preferential tissue accumulation of unconjugated genistein, which is the biologically active form. Intrathyroidal genistein

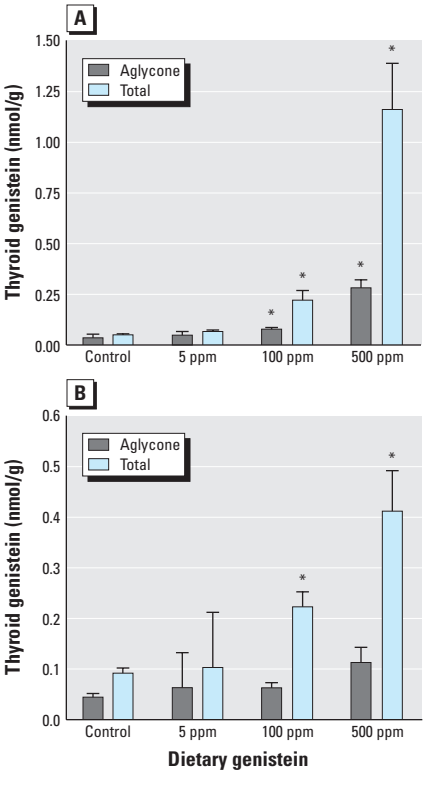


Figure 2. Dietary consumption of genistein increases intrathyroidal concentrations of genistein. The concentrations of genistein present in thyroid tissue, both total and aglycone, were determined using LC-ES/MS for female (*A*) and male (*B*) rats. Statistical significance relative to the respective control (*) was determined using Dunnett's test ($p < 0.05$). Data represent mean ± SE (*35*).

aglycone levels of 50–300 nM measured in this study are above concentrations that inactivate rTPO *in vitro* (Figure 1) (*35*).

Inactivation of rTPO by Dietary Genistein *in Vivo*

Given that the thyroid concentration of genistein was sufficient to inactivate TPO *in vitro*, TPO activity was assayed in rats fed the genistein-fortified diets. In both male and female rats, dose-dependent and significant decreases in rTPO activity were observed (Figure 3). Although 80% loss of rTPO activity was observed in high dose

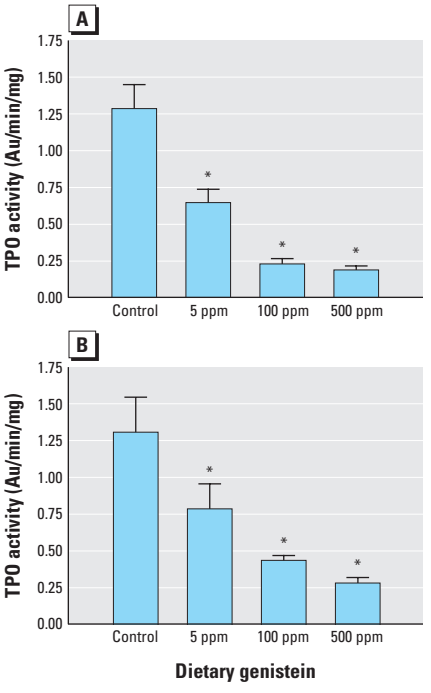


Figure 3. Dietary consumption of genistein decreases rat TPO activity. Microsomal rTPO activity was measured in thyroids from female (*A*) and male (*B*) rats using a spectrophotometric guaiacol oxidation assay. One-way analysis of variance demonstrated a significant treatment effect ($p < 0.05$) for both males and females, and statistical significance relative to the respective control (*) was determined using Dunnett's test ($p < 0.05$). Data represent mean ± SE (*35*).

(500 ppm) females, a dose 100-fold lower (5 ppm) also inactivated significant amounts of rTPO. That suggests that TPO inactivation can occur, even at very low dietary isoflavone levels. Furthermore, because thyroid genistein concentrations were sufficient to inactivate rTPO *in vitro*, the reductions seen in TPO activity could have been due to enzyme inactivation *in vivo*.

We found no evidence from LC-ES/MS analysis of serum or thyroids suggesting minimal formation of any of the iodinated genistein species previously characterized *in vitro* (*33*). This finding, coupled with the extensive TPO inactivation *in vivo*, suggests that TPO inactivation by covalent binding of

isoflavones predominates in the rat thyroid gland over competitive substrate iodination. This study with genistein appears to be the first in which chemically induced loss of TPO activity has been demonstrated both *in vitro* and *in vivo*.

Inactivation of rTPO by Dietary Soy *in Vivo*

A diet comparison study was also conducted in which rats were fed either a standard rodent diet (NIH 31) containing 5% soy meal and approximately 60 ppm total isoflavones or a soy-free basal diet (5K96) containing approximately 1 ppm total isoflavones (*35*). In soy, isoflavones occur as various glucoside conjugates (*42*), so this study addressed the issue of whether conjugation of dietary isoflavones produced effects different from genistein aglycone. TPO activity was reduced approximately 50% in male and female rats consuming the standard soy diet compared with rats fed the control diet (Figure 4), and the extent of reduction was consistent with the total isoflavone blood levels. The average serum concentrations of total genistein and daidzein from the NIH 31 diet were 0.35 ± 0.03 and 0.20 ± 0.02 µM, respectively, in males and 0.62 ± 0.05 and 0.25 ± 0.02 µM, respectively, in females (*43*). These results clearly showed that whether the dietary isoflavone is an aglycone or a glucoside conjugate has no effect on total serum isoflavones or on TPO inactivation. This result is consistent with previous studies that showed administration of genistein aglycone or conjugates had a minimal effect on the pharmacokinetics of total isoflavone absorption and elimination in rats, although small differences in the peak concentrations were found (*44*). The isoflavone content of typical open-formula rodent diets can range from <5 ppm up to 500 ppm (*45*). These findings suggest that additive effects on TPO activity between soy in rodent diets and exogenous chemicals being tested for carcinogenicity or other toxicities may confound the conclusions.

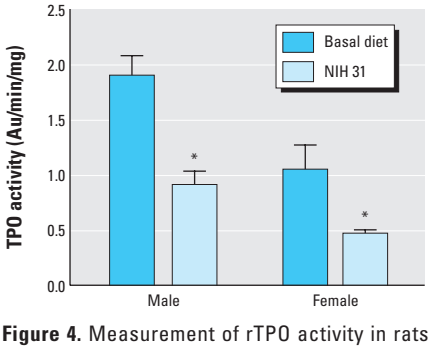


Figure 4. Measurement of rTPO activity in rats consuming either a standard soy-containing (basal) diet or a soy-free (NIH 31) diet. Microsomal TPO was isolated from female and male rats fed NIH 31 diet (~30 ppm each of genistein and daidzein) or the basal diet (~0.5 ppm each of genistein and daidzein) and rTPO activity measured spectrophotometrically. Data represent mean ± SE (*35*).

Absence of Hypothyroid Indicators in Rats Fed Genistein

Given the decreased TPO activity in rats on genistein-fortified or soy diets, a resultant hypothyroid state with decreased T_3/T_4 and increased TSH levels seemed likely. This was further reinforced by the known susceptibility of rats (particularly males) to antithyroid chemicals (*8*). However, analysis of T_3/T_4 and TSH in sera from all rats in both these studies (*35*) showed that no treated group was different from the untreated controls (data not shown). Consistently, gland weights and histopathology of thyroid sections were not different between the control and 500-ppm genistein groups in a parallel study (not shown). A recent study showed no significant thyroid histopathology in rats fed a 20% soy diet (*46*). These findings appear paradoxical given the prominent losses of TPO activity (Figures 3, 4). Unlike these results, humans consuming soy developed goiter and elevated TSH, albeit without changes in T_3/T_4 (*24*).

Synergism of Soy with Iodine Deficiency in Producing Hypothyroid Effects in Rats

The synergism in antithyroid properties of soy combined with iodine deficiency has been further elucidated by Ikeda et al. (*46*). They showed, as did Kimura et al. (*14*), that rats fed an iodine-deficient diet containing 20% defatted soy bean diet developed a severe hypothyroid state characterized by decreased T_4, increased TSH, increased thyroid weight, increased cell proliferation, and marked histopathological changes. Histopathology of the anterior pituitary showed that an unknown component of soy had a direct action on the pituitary gland.

To further investigate the ability of soy isoflavones to interact with iodine deficiency to produce hypothyroidism, effects on rats fed soy-free diets (with or without iodine) were compared with effects on rats fed the 20% defatted soy diet (*47*). In other groups, soy isoflavones (12–18% genistein aglycone, 12–18% daidzein aglycone, 2–4% glycitein aglycone) were added to soy-free diets at 0.2 and 0.04%. The 0.04% isoflavone diet had isoflavones at the same level as the 20% defatted soy diets. As before, the hypothyroid state in rats on the 20% defatted soy diet required iodine deficiency. However, neither group of isoflavone-supplemented rats showed hypothyroidism, whether iodine deficient or iodine replete. These results suggest that whole soy, but not isoflavones alone, is required to produce hypothyroidism in iodine-deficient rats. This is consistent with isoflavone inhibition of TPO synergistically interacting with an unidentified component(s) of soy to produce hypothyroidism in iodine-deficient rats. Additionally, other components of human or rat diets, or chemical exposures, may cause goiter in association with soy consumption.

Possible Effects of Soy on Human Thyroid Health

The total genistein concentrations in rat serum (Table 1) are similar to those in humans (Table 2), suggesting a similar tissue exposure. It reasonable to conclude that human isoflavone consumption could produce isoflavone levels in the thyroid (Figure 2) sufficient to inactivate human TPO, as seen in rats.

The failure to find hypothyroidism caused by genistein in rats, despite extensive inactivation of TPO (*35*), or by mixed isoflavone consumption (*47*) points to additional risk factor(s) necessary to induce hypothyroidism. In particular, iodine deficiency is necessary for soy to cause antithyroid effects in rats. Although the mechanism of this iodide effect is unknown, a significant literature supports this concept (*10,14, 46,47*). In humans, early findings showed that goiter in infants fed soy formula was reversed upon supplementation with iodine (*17*). Progression to a hypothyroid state may also be aided by biochemical impairment of hormone synthesis and metabolism, and/or exposure to environmental goitrogens, for example, sulfonamides, glucosinolates, cyanogenic glycosides, flavonoids (*48*), and persistent halogenated aromatic compounds. Nonetheless, we should be alert to the finding that soy-induced goiter and other hypothyroid indicators have been reported in humans in the absence of evidence for iodine deficiency (*24*).

Soy products are heavily marketed to postmenopausal women for relief of menopausal symptoms, despite the absence of consistent clinical data demonstrating any such benefit in human trials (*25*). However, of concern is that this is the same subgroup in which frank hypothyroidism and a subclinical hypothyroid state (*49*) are most likely to occur (up to 4 and 10%, respectively) (*50,51*). Further, the incidence of chronic autoimmune thyroiditis, the major risk factor for the development of hypothyroid disease in women (*50*), increases with age in women.

Dietary genistein causes a potent stimulation of T-cell– and B-cell–mediated immunity in rats (*36,52*), an effect found with other estrogenic compounds (e.g.,

p-nonylphenol, ethinylestradiol). In addition, suicide inactivation of TPO by dietary genistein in rats likely produces covalent binding of genistein to TPO. This modification could well be followed by TPO structural changes, especially in the three-dimensional shape and charge distribution. This could lead to a new antigenic form of TPO (a neoantigen) that could stimulate recognition by the immune system (*53*). Further support for this notion comes from the fact that anti-TPO is the major thyroid autoantigen circulating in human serum (*54*). Although the etiology of thyroid autoimmunity is unknown, the multitude of genistein effects in rats suggests that soy consumption could cause or exacerbate this illness.

Iodine deficiency is an emerging concern in elderly Americans. Consumption of iodized salt, the primary source of dietary iodine, may decrease with the desire or need to reduce the possible hypertensive effects of high salt intake. The data presented here suggest that elderly women need to be aware of, and monitored for, possible thyroid problems resulting from consumption of soy products. Those postmenopausal women who consume large amounts of soy products may be at higher risk.

Finally, information presented here also allows a new understanding of the increased incidence of autoimmune thyroiditis in children fed soy formulas reported by Fort et al. (*23*). That study has been criticized, and even dismissed, because children put on soy formula are thought to be more likely to have autoimmune disorders (e.g., food allergies, presence of viral gastroenteritis). This assumption, however, can be seriously questioned, because only 0.5–1.0% of American infants have true allergies, but about 20% are on soy formula. The alternative explanation, based on the findings here, involves stimulation of immune function by genistein, possible neoantigen formation through covalent binding of genistein to TPO, and increasing autoimmune disease prevalence. This hypothesis provides a plausible explanation for the observations of Fort et al. (*23*) and we hope will encourage further study of autoimmune thyroiditis in children consuming soy formula.

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