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REVIEW

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Isoflavones, substances with multi-biological and clinical properties

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Summary Isoflavones, rich in soybean, are currently receiving much attention because of their potential role in preventing and treating cancer and other human chronic diseases. The present review provides an overview of the recent results in this research field. Data from epidemiological reports and laboratories have shown that isoflavones have multi-biological and pharmacological effects in animals and humans. These include estrogenic and antiestrogenic effects, cell signalling conduction, as well as cell growth and death. Based on these properties, soy protein and isoflavones have been associated with reduced incidences of breast and prostate cancers, cardiovascular diseases or osteoporosis, and exhibit some other favorable effects. The mechanism through which isoflavones may exert the above-mentioned functions are not only based on the estrogenic properties of isoflavones, but also on their role as protein tyrosine kinase inhibitors, as regulators of gene transcription, modulators of transcription factors, antioxidants, as well as by altering some enzyme activities. However, to draw a clear conclusion regarding the clinical use of isoflavones further investigation would be required, although only a few effects of short- or long-term use of soy proteins are known in humans.

Key words Soy protein – Isoflavones – Estrogen receptor – Cancer – Cholesterol

Introduction

Plant-derived, nonsteroidal weakly-estrogenic compounds are defined as phytoestrogens, and may act as fungicides, deter herbivores, regulate plant hormones, and protect plants against ultraviolet radiation [1]. There are at least 20 phytoestrogen compounds found in nature. They have been identified in at least 300 different plants from more than 16 different plant families [2]. Plants related to human and animal food such as seasonings (garlic, aniseed, fennel, caraway, parsley), legumes (soybeans, chick peas, clover), grains (wheat, barley, rye, rice and oat), vegetables/herbs (carrots, potatoes, alfalfa, red clover), fruits (apples, pears, grapes, dates, pomegranates, cherries), and drinks (beer, coffee) also contain phytoestrogens. Chemically, phytoestrogens can be divided into several classes, for example, isoflavones, coumestans, and lignans [3]. Indol-3-carbinol, a hydrolysis product of glucosinolates, also has estrogenic activity [4]. The main feature of the chemical structure of isoflavones is strikingly similar to mammalian estrogens. While phytoestrogens act mainly by binding to the second subtype of estrogen receptor (ERβ), a higher binding affinity to the "classic" ERα has been identified for mammalian estradiol [5, 6]. For this reason, phytoestrogens can act either as estrogen agonists or as antagonists [7, 8]. Among the classes of phytoestrogens, isoflavones have been the most extensively researched. Therefore, the aim of

this paper was to review recent progress on isoflavone research.

Isoflavone sources, absorption and metabolism

Soybeans and soy products are a particularly abundant source of isoflavones. They contain approximately 0.2–1.6 mg of isoflavones/g dry weight [3]. Chick peas and other legumes, as well as clover, toothed medic, and bluegrass are other isoflavone sources [9]. The principal isoflavones found in soy proteins and soy foods are daidzein, genistein, and glycitein. Each of them is found in four chemical forms: the unconjugated form, or aglycone; the conjugated form, or glucoside (daidzin, genistin, and glycitin); acetylglucoside; and malonylglucoside. The soy isoflavones daidzein and genistein primarily appear in the form of their glucosides, daidzin and genistin, respectively [10, 11]. Processing and fermentation of the soybean is known to influence the forms of isoflavones [12]. The bioavailability and biological activities of different isoflavones also differ to some extent [13, 14]. Moreover, the estrogenic potency of equol is higher than its precursor, daidzein [8].

After ingestion, the conjugated form of isoflavones is hydrolyzed by intestinal β-glucosidases, which release the principal bioactive aglycones, daidzein and genistein. These compounds may be absorbed or further metabolized in the distal intestine with the formation of specific metabolites, such as equol and *p*-ethylphenol [11]. Three native β-glucosidases have been identified in humans [15]. The first is glucocerebrosidase, being a lysosomal enzyme which hydrolyses glucoceramide from endogenous membrane glycolipids. Another is lactase phlorizin hydrolase, which is a membrane-bound enzyme found in the brush-border of the small intestine, and is primarily responsible for hydrolysis. The third β-glucosidase is a broad-specificity cytosolic enzyme found in abundance in the liver, kidney, and small intestine of mammals. Some intestinal bacteria produce β-glucuronidases, which can deconjugate these isoflavone metabolites when they pass through the intestine [11]. The aglycones along with any bacterial metabolites are absorbed from the intestinal tract and transported via the portal venous system to the liver, where the isoflavones and their metabolites are efficiently conjugated with glucuronic acid (95 %), and to a lesser extent are found as sulfate conjugates [16]. They are then excreted in the urine or in the bile [14]. Some isoflavones undergo enterohepatic recycling. It has been proposed that intestinal metabolism is essential for their subsequent absorption and bioavailability in the body. However, Andlauer et al. [17] reported that genistin was partly absorbed without previous cleavage. Piskula et al. [10] also demonstrated that both aglycones and their glucosides are absorbed very fast. These results contradict the above assumption. The results from Izumi et al. [18] showed that the isoflavone aglycones were absorbed faster and in greater amounts than their glucosides in humans. The peak concentrations of isoflavones in blood are seen generally 4–8 h after dietary intake [11, 19]. Most of the daidzein and genistein are excreted in urine within the first 24 h after food intake [20, 21]. The rate of urinary excretion of daidzein was greater than that of genistein throughout the postmeal period [22]. Differences are observed in the elimination half-life for different studies. Watanabe et al. [23] found that after ingestion of 60 g baked soybean powder, the half-lives of plasma genistein and daidzein were 8.36 and 5.79 h, respectively. While with the same foods, from King and Bursill's [22] research, the elimination half-lives were 4.7 and 5.7 h for daidzein and genistein, respectively. More rapid elimination is observed for isoflavones in a liquid matrix than in a solid matrix [11].

In ruminant animals, the absorption of isoflavones takes place mainly in the rumen, where the gastrointestinal epithelium is the major site of metabolism. The liver contributes very little to the total degradation of isoflavones in ruminants [16]. Metabolism of isoflavones in pigs is not as well documented. The pig seems to differ markedly in comparison with ruminants regarding conjugation of equol. Only 50–70 % of equol was found in the conjugated form, whereas the corresponding figure for conjugated equol in plasma from cow and sheep is 95–99 %.

The presence of different populations of microflora in the human gut may influence the bioavailability of isoflavone phytoestrogens and causes wide inter-individual variation in isoflavone metabolite excretion [13, 24]. The reasons for the considerable inter-individual variation in isoflavone metabolism following the consumption of soybean isoflavones have not been fully elucidated. Recent data from a human intervention study of soy-containing food (low or high in isoflavones) showed that the proportion of energy from fat affects phytoestrogen excretion in the urine. Dietary fat intake decreases the capacity of gut microbial flora to synthesize equol [25]. Further investigations in phytoestrogen bioavailability of the different types of gut microflora are needed.

Isoflavones can be detected in many tissues of animals and humans. Yueh and Chu [26] reported the tissue distribution of daidzein in rats 15 min after intravenous injection of 40 mg daidzein/kg body weight. Daidzein concentration was found to be high in plasma, liver, lung, and kidney at about 30 μg/g wet weight; to be moderate in skeletal muscle, spleen, and heart at about 15 –20 μg/g wet weight; and to be low in brain and testis at about 2–5 μg/g wet weight. In another experiment rats were exposed to genistein at 5, 100 and 500 μg/g feed [27]. Tissues including brain, liver, mammary, ovary, prostate, testis, thyroid and uterus showed significant

dose-dependent increases in total genistein concentration. This research also found that the liver contained the highest amount of genistein while brain tissue accumulated less genistein, as compared to other tissues. Similar results for daidzein were obtained by Janning et al. [28], who found that the daidzein levels were usually three- to fivefold higher in the liver and kidney than in plasma.

Plasma concentrations of 50–800 ng/mL (approx. 0.2–3.2 μmol/L) were found for daidzein, genistein, and equol in adults consuming modest quantities of soy foods containing ~ 50 mg/d of isoflavones [8, 29, 30]. In response to the consumption of soy foods, blood isoflavone concentrations can reach ≤ 6 μmol/L [13]. When soy is consumed on a regular basis, plasma isoflavone levels far exceed normal physiological estradiol concentrations, which in men and women generally range between 40 and 80 pg/mL [8]. These observations led to the hypothesis that isoflavones would be biologically active, conferring health benefits that could explain the relatively low incidence of hormone-dependent diseases in countries in which soy is a dietary staple.

Biological effects

Hormonal effects

Since 1931 it has been known that soybeans contain relatively high concentrations of isoflavones [31]. Genistein glycoside was first isolated from soybeans by Walter [32]. However, it was unknown whether these compounds could have biological activity in animals until the recognition of the infertility syndrome in sheep correlated with the hormonal potency of isoflavones [33]. Isoflavones are structurally similar to mammalian endogenous estrogens [8], and thus may act as estrogen agonists or antagonists [34], depending on the isoflavone concentration, or the tissue of action. They act mainly by binding to the ERβ [5, 6], which was found to be expressed in many tissues, including the hypothalamus, pituitary gland, lung, and thymus.

Isoflavones have been shown to possess an estrogen hormone function. They have been shown to induce specific estrogen-responsive gene products and stimulate the genital tract of female animals. In rodents and rats, isoflavones were also found to stimulate mammary and uterine growth [35]. But compared to estradiol, the isoflavone estrogenic effects are weak. In the mouse uterine growth assay, genistein and daidzein are roughly 100,000 times less effective than estradiol [5].

The hormonal actions of isoflavones might explain epidemiologic observations of lowered risk for chronic diseases and menopausal symptoms in populations that consume soy. However, effects of soy consumption on hormonal metabolism have been inconsistent among most studies, probably as a result of methodological differences in subjects characteristics, study design, isoflavone form, dosage, and length of diet period. Very often, soy isoflavones have been provided as different soy protein sources (soy protein isolate, soy milk, textured vegetable protein) so that the resulting effects could not be direct proof for the action of isoflavones.

Soy isoflavones appear to affect the menstrual cycle and concentrations of reproductive hormones in premenopausal women. Cassidy et al. [36, 37] found that premenopausal women consuming 60 g textured soybean (containing 45 mg isoflavones) experienced a 2.5 d increase in the length of their follicular phase whereas no change was noted in women fed on a similar amount of soybeans from which the isoflavones had been chemically removed. Particularly noteworthy is the finding that serum follicle stimulating hormone and luteinizing hormone levels decreased significantly in response to the consumption of soybean isoflavones. It can not be fully concluded, however, from this study that isoflavones are responsible for the observed effects since ethanol treatment also extracts other bioactive compounds. Results similar to those found by Cassidy et al. [36, 37] were reported for premenopausal Japanese women by Nagata et al. [38]. However, a recent study failed to find an effect of soybean isoflavones on menstrual cycle length or estrogen levels [39]. In postmenopausal women, the effects of soy isoflavones on endogenous estrogen metabolism were shown to be less pronounced than in premenopausal women [40, 41].

Moreover, it was observed that dietary genistein exerts estrogenic effects upon the hypothalamic-pituitary axis in rats, increases plasma prolactin [35], and enhances both GHRH-stimulated cAMP accumulation and GH release in rat anterior pituitary cells [42]. Genistein and daidzein suppressed glucocorticoid and stimulated androgen production in cultured human adrenal cortical cells [43].

At high dosages, isoflavones may act as antagonists of estrogen. They have generally been reported to have lower binding affinity for estrogen receptors and a lower potency in producing estrogenic effects compared with 17β-estradiol. Thus, when isoflavones displace 17β-estradiol molecules, it can reduce the function of real estrogen [8]. At concentrations 100–1000 times that of estradiol (the probable levels in human plasma after regular isoflavone consumption), isoflavones may be able to compete effectively with endogenous mammalian estrogens, bind the ERs, and prevent estrogen-stimulated growth in mammals [5]. This may also result in interference with the release of gonadotropins and interruption of the feedback-regulating system of the hypothalamus-pituitary-gonadal axis. Genistein and coumestrol have been shown to competitively suppress the binding of 17β-(^{3}H)estradiol to ERα when added to rat and human mammary tumor tissue. Daidzein and equol have been

demonstrated to compete effectively with 17β-(^{3}H)estradiol for rat uterine estrogen nuclear type II binding site (bioflavonoid receptor) with a 50% inhibitory concentration (IC_{50}) of 1–10 μM [44].

In summary, soy protein consumption appears to exert various hormonal effects. But, the resulting health benefits are of uncertain clinical significance. Further research is required to determine whether the responsible components are the isoflavones or some other soy constituents.

Regulating sex hormone receptors at the transcription level

Cotroneo et al. [45] found that at pharmacological concentrations (500 μg/g body weight), genistein decreased ERα mRNA levels in the rat uterus. It was also found that daidzein is capable of down-regulating androgen receptor and ERα mRNA expression significantly in rat uteri [46]. Our recent research has demonstrated that feeding daidzein to sows late in their pregnancy can markedly inhibit ERβ mRNA levels in the hypothalamus of newborn piglets. No changes were detected in the pituitary [47] indicating the possible central effects of daidzein on the neuroendocrine system. Kuiper et al. [5] found genistein and daidzein stimulated estrogen-dependent receptor gene activity at concentrations ranging from 10–1000 nM in cell cultures. Interestingly, some results indicated soy isoflavones increase nerve growth factor mRNA and brain-derived neurotrophic factor mRNA in rats [48, 49].

Influence cell signalling

Isoflavones, particularly genistein, can regulate the cell signalling conduction from receptor expression to cytoplasmic downstream signalling. Pharmacological doses of genistein (500 μg/g body weight) directly inhibited EGF receptor expression in the rat uterus and vagina [50]. Dalu et al. [51] also reported that genistein (1 mg/g diet) can downregulate both EGF and ErbB2/Neu receptors in the rat prostate with no apparent adverse toxicity to the host. Since Akiyama et al. [52] found that genistein inhibits tyrosine protein kinase activity, there have been more than 2000 published articles on this subject. Many of the peptide growth factor signal transduction pathways that were implicated in certain cancers involve the action of tyrosine kinases. Therefore, a circulating tyrosine kinase inhibitor, such as genistein, may have beneficial effects in the treatment of cancer. In androgen-independent human prostate carcinoma DU145 cells, genistein significantly inhibited the transforming growth factor (TGF)-α-caused activation of membrane receptor erbB1, followed by inhibition of downstream cytoplasmic signaling target Shc activation [53]. Recently, genistein has been shown to alter ion channel function of culture cells. Genistein (50 μM) reversibly reduced the peak currents of the A-type voltage-gated potassium channel in cloned Chinese hamster ovary cells [54] and inhibited Ca^{2+} fluxes in rat pituitary cells [55]. Similar results were also obtained for daidzein in the above mentioned investigations, although the effect of daidzein was weaker than that of genistein.

Cell proliferation, animal growth and development

Most isoflavone studies on cell proliferation were performed using estrogen-dependent human breast carcinoma MCF-7 cells. The results displayed biphasic effects: stimulation of growth at low concentrations and inhibition at high concentrations. Hsu et al. [56] reported that cell growth was stimulated at a daidzein concentration of 0.25 μg/mL whereas the addition of daidzein at concentrations > 25 μg/mL significantly inhibited cell growth in a dosage-dependent fashion. Also, Wang and Kurzer [57] showed that genistein and biochanin A, at 0.1–10 μM, induced cell DNA synthesis 150 –235%, while at 20–90 μM, inhibited DNA synthesis by 50%. Similar results for genistein were also reported by Wang et al. [58]. Using the differential display reverse transcriptase polymerase chain reaction assay, Hsu et al. [56] demonstrated that the growth inhibitory effects of daidzein might be mediated through a block at the G1 stage of the cell cycle.

Soy and alfalfa are used as protein sources in most animal diets, and therefore the animals ingesting these diets are continually exposed to isoflavone compounds. Moreover, isoflavones can freely pass the placental barrier. In humans the isoflavone concentrations in the neonate are similar to those in maternal plasma [59]. Research has reported that isoflavones at concentrations found in a standard natural-ingredient diet may affect the sexual differentiation of female rats *in uteri* [6]. At high dietary concentrations (100 mg/100 g feed), genistein decreased body-weight gain, while increasing the uterine:body weight ratio of female rats [6]. There are a few publications about the effects of isoflavones on body growth, especially on farm animal growth. Soy isoflavones decreased fat and increased lean content in barrows when fed within the dietary concentrations found in typical corn-soybean meal diets but not when fed to gilts at concentrations above those present in corn-soybean meal diets [60]. Liu et al. [61] demonstrated that daidzein fed to pregnant sows promoted fetal growth, improved sow milk production and affected the postnatal growth. Other results from rats report the reverse effect [6, 62]. Our recent experiments on sows confirmed the results of Liu et al. [61]. We found when sows were fed with daidzein the expression of IGF–1R

gene in *longissimus* muscle of newborn piglets was enhanced markedly [47]. This result suggests that daidzein may one way influence fetal growth is via up-regulation of IGF–1R expression in skeletal muscle.

Pharmacological and therapeutic effects

Anti-cancer

Several papers have reviewed the potential roles of soy or its isoflavones in decreasing the risk of cancer [63–66]. Most of the support comes from epidemiological studies. Epidemiological data suggest that a diet rich in isoflavones provides protection against several forms of cancer, particularly those that are hormone-dependent, such as breast, prostate, and lung cancer [66]. Tofu consumption has been negatively correlated with prostate, breast, and lung cancer, as well as leukemia in China and Japan [67], where people consume more soybean products than people in western countries. Furthermore, Asian people who have emigrated to Western countries and who generally adopt the dietary habits of the host country are at an increased risk for breast and other hormone-dependent cancers compared to those in their original countries [68]. *In vitro* data have demonstrated that isoflavones inhibit cancer cell growth, including prostate cancer cells [69, 70] and MCF–7 human breast cancer cell line [71]. There are literally hundreds of *in vitro* studies showing that genistein inhibits the growth of a wide range of both hormone-dependent and hormone-independent cancer cells with IC_{50}-values between ~5 and 100 μmol/L (2–25 μg/mL) [reviewed in 72]. The concentration of genistein required to inhibit angiogenesis *in vitro* was reported to be higher than the genistein concentration likely to be achieved *in vivo* [72, 73]. However, it has been found that isoflavones *in vitro* can be also active at physiologically relevant concentrations (< 5–6 μmol/L) [44, 74].

In animal studies, neonatal injections of pharmacologic doses of genistein have been shown to suppress the development of dimethylbenzanthracene (DMBA)-induced mammary adenocarcinomas in rats [75]. So far, there have been very few human studies to reveal direct evidence that soy intake or isoflavones may protect against breast cancer.

Xu et al. [76, 77] suggested that isoflavones may exert cancer-preventive effects by decreasing estrogen synthesis and altering metabolism away from genotoxic metabolites toward inactive metabolites. Recently, Lu et al. [68] found that daily consumption of the soya diet (providing 113–207 mg/day of total isoflavones) reduced circulating levels of 17β-estradiol by 25 %, and of progesterone by 45 % compared with levels during the control diet period for healthy and regularly cycling women.

It has become apparent that the anti-cancer mechanisms of isoflavones are not exclusively via the estrogen receptor. *In vitro* studies have revealed that numerous mechanisms may be involved. One is via inhibiting protein tyrosine kinases (see above). Many of the peptide growth factor signal transduction pathways have been proven to be implicated in certain cancer development. Therefore, a circulating tyrosine kinase inhibitor such as genistein may have beneficial effects in the treatment of cancer [67]. However, in several cell lines, genistein did not alter tyrosine phosphorylation of the EGF receptor or other tyrosine kinase substrates [78]. Dalu et al. [51] reported genistein inhibited the expression of the EGF receptor in the rat dorsolateral prostate, suggesting that genistein has its effects through transcriptional processes rather than directly on tyrosine kinase activity. Therefore, it is possible that the variable effects of isoflavones in estrogen-sensitive tissues may depend on the production of paracrine and autocrine growth factors that cause proliferation of cells not expressing ERα or ERβ [78]. Other anti-cancer mechanisms of isoflavones may include inhibition of 3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, 5α-reductase and aromatase [63, 66, 79], followed by affecting the level of active steroid hormones. Isoflavones also inhibited DNA topoisomerase I and II activity [80–82], which were predicted to cause DNA damage. The transcription factor p53 currently has become the most important tumor suppressor [83]. It has been shown that genistein induced the up-regulation of p53 protein [84]. More recently, it was suggested that genistein may inhibit cell growth by both increased expression [85] and production [86] of transforming growth factor (TGF) β1 signaling pathways. Hsu et al. [56] suggested that the inhibitory effects of biochanin A, the precursor of daidzein, on human breast cancer growth are linked to a decreased level of inducible nitric oxide synthesis and thus an inhibition of the production of nitric oxide and the later induction of cell apoptosis. Davis et al. [87] reported that genistein induced apoptosis by inactivation of NF-κB, providing a mechanism by which genistein promotes cell death. Another mechanism to partially explain the anti-cancer activity of isoflavones involves their ability to inhibit angiogenesis, or new blood vessel growth, which is required for tumor growth. Genistein is capable of blocking this process [88, 89]. Today, one of the most exciting approaches to cancer treatment involves angiogenesis inhibitors, such as a synthetic agent endostatin [90], and genistein may be its natural counterpart.

It is worth noting, however, that the preventative effects of soy are not as definite as commonly believed. Certain references describe adverse effects of isoflavones in relation to breast cancer. Obviously, the isoflavones dose is crucial to increase or decrease cancer risk (see "cell proliferation"). *In vitro*, studies have demonstrated that genistein enhanced the proliferation

of estrogen-dependent human breast cancer (MCF-7) cells at concentrations as low as 10 nM, with a concentration of 100 nM achieving proliferative effects similar to those of 1 nM estradiol. At higher concentrations (> 20 μM), genistein inhibits MCF–7cell growth. Moreover, dietary genistein (750 ppm) was also able to stimulate mammary gland growth and enhance the growth of MCF–7 cell tumors in athymic mice *in vivo* indicating that genistein acted as an estrogen both *in vitro and in vivo* [91]. In another *in vivo* experiment with athymic mice containing xenografted MCF–7 tumors, it was shown that feeding high dosages of genistein (750 ppm) or genistin (1200 ppm) resulted in increased tumor growth rates and cell proliferation. Removal of the isoflavones from the diet resulted in tumor regression [92]. Soy diets containing varying amounts of genistein stimulated the growth of estrogen-dependent (MCF–7) tumors in a dose-dependent manner [93]. Similar results were also reported by Santell et al. [73].

Peeters et al. [94] estimated that the real protective effect of phytoestrogens may be smaller than expected or only limited to premenopausal women. Finally, meta-analysis of Trock et al. [95] demonstrated that recommendations for women to increase their soy intake to prevent breast cancer or prevent its recurrence are premature and that larger, more rigorously controlled studies are required. New data from an epidemiological study suggest a positive association between soy intake and percent mammographic densities among a population of women from different ethnic groups living in Hawaii [96]. Nutritional effects before puberty were presumed as important as for breast development and cancer risk. Similar conclusions were drawn by Lamartiniere et al. [97] and Murrill et al. [98].

Moreover, soy intake in premenopausal women may increase breast cancer risk by elevating the levels of prolactin. Preliminary data suggested positive relations between estrogenic effects, plasma prolactin levels, and breast cancer risk [35, 99].

Lowering the risk of cardiovascular diseases

Many investigations have demonstrated that soy protein inhibits cardiovascular diseases and reduces atherosclerosis risk in animals and humans [64, 100, 101]. The beneficial effects of soy are thought to be caused primarily by isoflavones and appear to be mediated by many mechanisms. Most researchers consider that these effects result from a reduction of plasma low density lipoprotein (LDL) cholesterol [102–104] and triglyceride concentrations [104, 105]. Hamilton and Carroll [106] were the first to report that soy protein lowered plasma cholesterol in hypercholesterolemic rabbits. Many reports confirmed these findings to some extent. In October 1999, the US Food and Drug Administration acknowledged the health claim regarding the beneficial effects of soy-based foods on a heart-healthy diet. The agency reviewed research from 27 studies that showed soy protein's value in lowering levels of total cholesterol and low-density lipoprotein [107]. Other mechanisms for inhibiting cardiovascular disease include lowering diastolic blood pressure in women [108], improving vascular and endothelial cell function [109, 110]. There is an indication that soy protein may also inhibit platelet activation and aggregation and reduce the amount of serotonin in the platelets [111]. However, this result should be considered carefully since alcohol-washed, isolated soy protein was used.

Studies with transgenic mice [112] and in the human liver [113] suggest that the benefits of soy protein on cholesterol lowering may be mediated through an up-regulation of LDL-receptor activity, thus, providing a novel mechanism of plasma cholesterol reduction different from currently available diets and hypolipidemic drugs. Otherwise, the results from some researchers indicated that the hypocholesterolemic effects of soy protein may function by influencing lipid metabolism through altering lipid-related gene expression. For example, isolated soy protein and isoflavone-containing extract reduced hepatic apolipoprotein A–1 mRNA levels in gerbils [102]. The results of Tikkanen et al. [114] showed that intake of soy protein containing 60 mg isoflavones per day may provide protection against oxidative modification of LDL. The oxidative modification of LDL particles is considered to be a prerequisite for the uptake of LDL by macrophages in the artery wall, which is an initial step in the formation of atheroma. Thus, this may be one of the mechanisms of soy protein inhibition of atherosclerosis. Recent studies suggested that estrogen-induced cardiovascular protection might be mediated by an increased synthesis of vascular nitric oxide [71].

Currently, the mechanisms associated with soy's beneficial effects on cardiovascular health are not fully understood. All of the above mechanisms may potentially contribute to the observed beneficial effects. It remains unclear which components of soy protein contribute to its protective effects. It is possible that soy substances other than isoflavones such as saponins, phytic acid, protein components, amino acid composition or a protein-isoflavone interaction may be involved in the multifarious processes. The ability of saponins to lower cholesterol in some species is especially well known [115]. In a recent study, the anti-atherogenic effects of an isoflavone aglucone-rich extract compared with a saponin-rich extract (both without soy protein) in cholesterol-fed rabbits were investigated. It was shown that the isoflavone aglucone-rich extract inhibited progression of atherosclerosis while the saponin-rich extract was without effect [116]. New data demonstrating that milk protein causes a comparable effect on lowering total- and LDL-cholesterol as soy protein with and without

isoflavones are of interest [117]. Studies investigating the effects of soy protein and its constituents, especially with clinical conditions, are needed to further clarify these observations. Evidence in humans is still scanty [111].

Other diseases

Estrogen is used in hormone replacement therapy to prevent menopausal symptoms and osteoporosis in postmenopausal women [118]. But estrogen has been proven to be associated with an incidence of breast and endometrial cancer [119]. This relationship has severely hampered the clinical use of estrogen. Therefore, there is growing interest to use isoflavones as a potential alternative to the estrogens in hormone replacement therapy. Observational studies have shown a lower incidence of menopausal symptoms and osteoporosis in Asian women who have a diet rich in soy products [63, 65, 120]. In clinical studies, menopausal women who consumed isoflavone-enriched foods have alleviated symptoms associated with hot flashes [121, 122]. Another study has reported no beneficial effects of isoflavone on hot flashes [123]. Thus, data are currently insufficient to draw definitive conclusions regarding the use of isoflavones for the treatment of menopausal symptoms.

Osteoporosis is characterized by a loss of bone mass usually associate with aging, due to increased bone resorption and reduced bone formation. Approximately 1 million Americans suffer fragility fractures each year at a cost of over 14 billion dollars [118]. Thus, the prevention of osteoporosis is a major public health concern. The beneficial effects of estrogen replacement therapy (ERT) on prevention of postmenopausal osteoporosis are well known. But owing to the above addressed reasons, more researchers have begun turning to isoflavones as an alternative therapy. Data from animal studies suggest that isoflavones could prevent bone loss that occurs as a result of estrogen deficiency, such as in ovariectomy rats [124–127]. Results from Yamaguchi's group have shown that daidzein and genistein stimulated osteoblastic bone formation [128–130], and inhibited osteoclastic bone resorption [131]. Data available from human studies about the effect of isoflavones on osteoporosis are limited. Potter et al. [132] showed a dose between 50 and 90 mg per day seems to be needed to show a skeletal benefit in postmenopausal women. Recently, Alekel et al. [133] reported soy isoflavones attenuated bone loss from the lumbar spine in perimenopausal women. Somekawa et al. [134] revealed consumption of soy products is associated with increased bone mass in postmenopausal Japanese women. However, other investigations failed to find the bone-repairing effect of isoflavones in postmenopausal women [135, 136]. Therefore, the impact of genistein and daidzein on bone loss appears to be minimal. However, one or more of the isoflavone metabolites may prove to be a clinically useful agent in the prevention and treatment of osteoporosis.

Moreover, there are results suggesting that midlife tofu consumption accelerates brain aging [137]. Higher midlife tofu consumption was independently associated with indicators of cognitive impairment and brain atrophy in late life. It was hypothesized that isoflavones in tofu may cause, at least in part, a neurodegenerative process.

Immune system

It is well known that estrogen has an important effect on the immune system. For example, most autoimmune diseases are more common in women than in men and quite frequently begin under conditions when estrogen levels change dramatically, e. g., during puberty, menopause, or pregnancy [138, 139]. The exact mechanisms involved in these metabolic processes have yet to be determined. The gastrointestinal tract and the immune system have often been overlooked, and not been considered as targets of estrogen. However, ERβ has been found to be very highly expressed in the human thymus and the gastrointestinal tract. Therefore, some of the immuno-modulatory effects of estrogen might be mediated via ERβ. Daidzein, *in vitro*, has been proven to increase the activation of murine lymphocytes [74]. In another *in vitro* study of Zhang et al. [44], it was shown that isoflavone glucuronides might not only compete with endogenous estrogen to inhibit estrogen-dependent proliferation of cancer cells. They are also able to activate natural killer cells to potentially increase the immune defenses of the body against cancer at nutritionally relevant concentrations.

In mice, it was found that daidzein at high dosages (20 and 40 mg/kg) enhanced several immunologic functions [140]. Our recent results showed that daidzein is inclined to promote the IGF–1R gene expression in thymus of pigs. Moreover, the sows given daidzein delivered more living piglets than sows without daidzein feeding [47]. Isoflavones have also demonstrated an anti-inflammatory potential in various animal models, including chronic ileitis [141], inflammation-induced corneal neovascularization [142], and ischemia reperfusion injury [143]. However, any potential anti-inflammatory benefit of an isoflavone diet needs to be balanced by the possibility that such dietary modifications may also be detrimental. For example, data indicated that a soy extract containing mixed isoflavones results in reduced antigen-induced eosinophilia in the lung in the guinea pig model with asthma [144]. This effect was accompanied by an increase in antigen-induced leakage of protein into the airspace.

Cellular and molecular mechanisms of isoflavone effects

It has become apparent from the diversity of isoflavone properties that no single action can explain many of the effects of isoflavones. A growing body of literature suggests that isoflavones exert multifunctions through genomic and nongenomic mechanisms of cellular regulation. First, isoflavones have a similar structure to estradiol and are capable of binding to the two estrogen receptors, ERα and ERβ. Secondly, isoflavones can interact with membrane proteins (receptors) and exert an effect that is expressed through secondary messengers in the cytoplasm [64].

Since discovery of a second estrogen receptor, ERβ [145], it is necessary to re-evaluate the molecular basis for the action of estrogen and its agonist. Structurally, ERβ is highly homologous to ERα in the DNA binding domain (> 95% amino acid identity) but shows only 55% homology in the ligand binding domain [138]. These structural differences lead to different relative binding affinities in ligand binding assays. Compared with ERα, isoflavones have a greater relative binding affinity to ERβ [5, 6, 146], while estradiol binds to ERα and ERβ with equal affinity. Studies by structural biologists [147] demonstrated that genistein is completely buried within the hydrophobic core of the protein and binds in a manner similar to 17β-estradiol. However, in the ERβ-genistein complex, the activation-function (AF)–2 helix (H12) does not adopt the distinctive "agonist" position but instead lies in a similar orientation to that induced by estrogen antagonists. Such a specific helix is consistent with genistein's partial agonist character in ERβ [148, 149].

Early observations showed the rapid affect of steroid hormones on cardiovascular, central nervous, and reproduction functions [150]. These phenomena are incompatible with traditional steroid hormone action, i.e., by triggering genomic events. Therefore, some cellular effects of steroids could occur via nongenomic mechanisms [151]. There are recent findings from cell lines that ERα and ERβ can associate with the G protein and protein kinase A (PKA) to activate many of the intracellular cascades [152]. For example, genistein potentiated GHRH-stimulated cAMP accumulation in a concentration-dependent manner [42]. Estrogen membrane receptors have been detected in GH3/B6 rat pituitary tumor cells by antibodies [153]. Wehling [150] predicted that in the near future the cloning of the cDNA for the first membrane receptor for steroids should be achieved. Once a G-protein-coupled ER has been cloned and the cellular cascades associated with its activation identified, it will be possible to characterize the multiplicity of actions of estrogen and isoflavones.

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