# Isoflavones Made Simple – Genistein's Agonist Activity for the Beta-Type Estrogen Receptor Mediates Their Health Benefits

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## Abstract

Soy isoflavones, the focus of much research and controversy, are often referred to as "weak estrogens". In fact, genistein is a relatively potent agonist for the recently characterized beta isoform of the estrogen receptor (ERbeta). The low nanomolar serum concentrations of unconjugated free genistein achieved with high-nutritional intakes of soy isoflavones are near the binding affinity of genistein for this receptor, but are about an order of magnitude lower than genistein's affinity for the "classical" alpha isoform of the estrogen receptor (ERalpha). Moreover, these concentrations are far too low to inhibit tyrosine kinases or topoisomerase II, *in vitro* activities of genistein often cited as potential mediators of its physiological effects. The thesis that these physiological effects are in fact mediated by ERbeta activation provides a satisfying rationale for genistein's clinical activities. Hepatocytes do not express ERbeta; this explains why soy isoflavones, unlike oral estrogen, neither modify serum lipids nor provoke the prothrombotic effects associated with increased risk for thromboembolic disorders. The lack of uterotrophic activity of soy isoflavones reflects the fact that ERalpha is the exclusive mediator of estrogen's impact in this regard. Vascular endothelium expresses both ERalpha and ERbeta, each of which has the potential to induce and activate nitric oxide synthase; this may account for the favorable influence of soy isoflavones on endothelial function in postmenopausal women and ovariectomized rats. The ERbeta expressed in osteoblasts may mediate the reported beneficial impact of soy isoflavones on bone metabolism. Suggestive evidence that soy-rich diets decrease prostate cancer risk, accords well with the observation that ERbeta appears to play an antiproliferative role in healthy prostate. In the breast, ERalpha promotes epithelial proliferation, whereas ERbeta has a restraining influence in this regard – consistent with the emerging view that soy isoflavones do not increase breast cancer risk, and possibly may diminish it. Premenopausal women enjoy a relative protection from kidney failure; since ERbeta is an antagonist of TGF-β signaling in mesangial cells, soy isoflavones may have nephroprotective potential. Estrogen also appears to protect women from left ventricular hypertrophy, and recent evidence suggests that this effect is mediated by ERbeta. In conjunction with reports that isoflavones may have a modestly beneficial impact on menopausal symptoms – perhaps reflecting the presence of ERbeta in the hypothalamus – these considerations suggest that soy isoflavone regimens of sufficient potency may represent a safe and moderately effective alternative to HRT in postmenopausal women. Further clinical research is required to characterize the impact of optimal genistein intakes on endothelial and bone function in men. Studies with ERbeta-knockout mice could be helpful for clarifying whether ERbeta does indeed mediate the chief physiological effects of low nanomolar genistein. S-equol, a bacterial metabolite of daidzein, has an affinity for ERbeta nearly as high as that of genistein; whether this compound contributes meaningfully to the physiological efficacy of soy isoflavones in some individuals is still unclear.

**Physiological Concentrations of Free Genistein Activate Estrogen Receptor-Beta**

The key to understanding the health-protective potential of soy isoflavones may have been provided by Kuiper and colleagues, who first established the existence of a "novel" estrogen receptor, now known as estrogen receptor beta (ERbeta) to distinguish it from the "classical" estrogen receptor alpha (ERalpha).[1;2] These workers assessed the affinity of these receptors for a range of xenobiotic and phytochemical estrogenic compounds, including the soy isoflavones.[3;4] They established that genistein has agonist activity for both ERalpha and ERbeta, but that genistein's affinity for ERbeta is considerably greater; genistein's affinities for Erbeta and ERalpha were determined to be 8.4 nM and 145 nM, respectively. For daidzein, the corresponding values were 100 nM and 420 nM, indicative of its much lower affinity for these receptors. At saturating concentrations, both genistein and daidzein could interact with either of these receptors to activate transcription from estrogen response elements, at least as effectively as the physiological ligand 17β-estradiol.

A number of subsequent studies have examined genistein's comparative abilities to bind to and promote transcription from the two estrogen receptor isoforms.[5-12] Although the absolute values obtained in these studies differ, genistein's binding affinity for ERbeta consistently emerges as 7-30-fold greater than its affinity for ERalpha; this is paralleled by genistein's ability to activate transcription with ERbeta at a lower concentration than with ERalpha. As a rule, ERbeta-mediated transcription is approximately half-maximal at a genistein concentration of 10 nM, whereas ERalpha-mediated transcription is minimal at this concentration, only becoming substantial as genistein rises above 100 nM.

One recent study has examined the impact of isoflavones on the rate at which the ER isoforms bind to the estrogen response element in DNA; they determined the isoflavone concentration which would increase this binding rate by 50%.[13] For genistein, this value was determined to be 30 nM for ERbeta and 15 μM for ERalpha – once again indicative of marked selectivity for ERbeta. The corresponding values for daidzein were 350 nM for ERbeta and >300 μM for ERalpha. For equol – which can be generated from daidzein in the GI tract by bacterial reductive activity – the values were 400 nM and 3.5 μM.

Some of the key effects of estrogen result not from transcriptional activation at estrogen response elements, but from transcriptional repression of certain promoters that bind NF-kappaB, such as the IL-6 promoter.[14] This latter effect reflects interaction of the activated estrogen receptor with NF-kappaB in a manner that does not entail binding of the estrogen receptor to DNA. Genistein has been shown to activate ERbeta such that it is capable of inhibiting the TNF response element; this effect was half-maximal at a genistein concentration of only 8.5 nM.[7] In contrast, in cells overexpressing ERalpha, only a moderate transrepression was seen with a genistein concention of 1 μM.

Estrogen receptors can also influence the transcriptional activity of AP-1, Sp1, and certain c-AMP response elements, through interactions that do not entail direct binding to

DNA.[15-17] In particular, agonist-activated ERalpha often increases the transcriptional activation mediated by AP-1, possibly by binding to coactivators that interact with fos/jun.[15;18] In contrast, activated ERbeta has the opposite effect, suppressing AP-1-mediated transcription.[15;19-22] Since AP-1 exerts various pro-proliferative effects, these findings may help to rationalize the opposing effects of ERalpha and ERbeta on cell proliferation in certain tissues, as cited below. In particular, ERalpha activates the cyclin D1 promoter through its AP-1 and c-AMP response elements, whereas ERbeta has a suppressive effect in this regard.[21] These considerations suggest that low nanomolar concentrations of genistein may have the potential to exert certain effects opposite to those of the classical estrogen receptor - including anti-proliferative effects.

The relevance of these findings becomes evident when one considers the plasma genistein concentration in subjects who habitually consume a soy-rich diet. Adlercreutz et al. reported that the mean concentration of total genistein (free plus conjugated) was 276 nM in Japanese men; however, only about 4% of this was in free or sulphated form, the balance consisting of the glucuronate conjugate which presumably has limited intracellular access.[23;24] More recent studies have specifically measured unconjugated plasma isoflavones; one of these found that unconjugated genistein constituted only about 1.1-1.5% of the total plasma pool of genistein – the higher percentage being observed transiently in the first 2 hours after soy ingestion.[25] The low content of unconjugated isoflavones is presumed to reflect rapid hepatic glucuronidation of these compounds. Since total serum genistein concentrations of around 1.5 μM are noted during prolonged supplementation with high-physiological doses of genistein,[26] this would correspond to an unconjugated genistein concentration of about 20 nM if 1.3% of total genistein were in free form. After several subjects ingested a meal providing 125 g (dry weight) of whole soybeans, free serum genistein rose to about 20-40 nM within about 2 hours, persisting at this level at 8 hours, but returning to baseline after 24 hours.[27] About half of the unconjugated genistein in serum is bound to serum proteins, so the effective concentration available to cells may be about 50% of the measured total concentration;[28] in other words, the physiological impact of 20 nM serum genistein may be comparable to that observed with 10 nM genistein *in vitro*.

These findings encourage the speculation that high physiological serum levels of free genistein – i.e. those achievable by ingesting a soy-rich diet – will achieve ample activation of ERbeta, but only minimal or modest activation of ERalpha. As we shall see, this thesis appears capable of rationalizing both the safety and the physiological benefits of dietary genistein.

**Irrelevance of Other Suspected Effects**

Much of the speculation regarding the physiological effects of isoflavones makes reference to in vitro studies in which genistein has been shown to inhibit tyrosine kinases or topoisomerase II, or to modulate activation of mitogenic signaling pathways in cultured cells. The effects of genistein on tyrosine kinase or topoisomerase activity require concentrations well into the micromolar range.[29;30] Similarly, the great majority of studies showing that genistein is a signal modulator in cells have used micromolar

concentrations of this agent. These effects thus have no conceivable relevance to the physiological impact of genistein. To the best of my knowledge, activation of estrogen receptors is the only effect of genistein that has been documented in the low nanomolar range.

There is a recent report that genistein, as well as daidzein and biochanin A, have agonist activity for the so-called estrogen-related receptors (ERRs).[31] These receptors are structural relatives of genuine estrogen receptors, and can activate transcription from estrogen response elements, but do not bind estrogen, and possess constitutive activity. Although isoflavones can bind to ERR and modestly enhance their transactivational activity, this effect is minimal and statistically insignificant at a genistein concentration of 1μM – nor are daidzein or biochanin A much more active in this regard. Thus, interaction of soy isoflavones with ERRs is unlikely to be of physiological significance.

**Soy Isoflavones Have No Hepatic Effects**

Studies in rats, primates, and humans demonstrate that hepatocytes express ERalpha, but not ERbeta.[32-36] Many of the notable physiological effects of oral estrogen are mediated in the liver. Thus, oral estrogen lowers LDL cholesterol, raises serum triglycerides, boosts synthesis of angiotensinogen and sex hormone-binding globulin, and decreases hepatic production of IGF-I – effects which are thought to reflect direct estrogenic activity in hepatocytes.[37-44] Moreover, oral estrogen up-regulates thrombotic mechanisms by modulating hepatic production of a range of plasma proteins which regulate thrombosis. Thus, oral estrogen increases plasma concentrations of clotting factors VII and IX, activated protein C, and C-reactive protein, while decreasing those of antithrombin, proteins C and S, and tissue factor pathway inhibitor.[45-49] These effects are much less substantial when estrogens are administered transdermally; this is thought to explain the observation that risk for venous thromboembolism is increased far more by oral estrogen than by transdermal estrogen.[47;50] Since physiological concentrations of genistein can be expected to have only a modest impact on ERalpha activity, it is not surprising that none of these effects are observed when soy isoflavones are ingested.[51-56]

Gallstone risk is higher for women than men, and this has been traced to the fact that activated ERalpha boosts cholesterol output to the bile. In mice, ERalpha-selective agonists, but not ERbeta-selective agonists, have this effect.[57] It can be deduced that soy isoflavones will not increase risk for gallstones.

The modest impact of soy protein-rich diets on elevated LDL cholesterol in some studies presumably reflects replacement of "high-quality" animal protein (such as casein) with "lower-quality" plant protein.[58] Indeed, Sirtori, who first established the utility of soy protein-based diets for cholesterol reduction, pointed out that the soy protein isolate he used to first demonstrate this effect was devoid of isoflavones![59] He maintained that the utility of his regimen was contingent on replacing animal protein with plant protein.[60] The studies which document reduction of elevated LDL cholesterol with supplemental soy protein have typically used comparable intakes of milk protein for the placebo

group;[61] there is little evidence that simply adding soy protein to a diet that remains high in animal protein will lower elevated LDL.

While it may be disappointing to concede that soy isoflavones cannot lower LDL cholesterol, or diminish cancer risk by suppressing plasma IGF-I, it is nonetheless comforting to realize that isoflavones will not increase thrombotic risk in the way that oral estrogens do. Conceivably, the prothrombotic hepatic effects of oral estrogen are largely if not wholly responsible for the unanticipated increase in risk for myocardial infarction observed during recent prospective trials of oral hormone replacement therapy – despite the favorable influence of estrogen activity on vascular endothelium and LDL cholesterol.[62]

**No Uterotrophic Activity**

Physiological concentrations of soy isoflavones can also be expected to be safe for the uterine endometrium, as the uterotrophic effect of estrogens appears to be mediated solely by ERalpha. This has been demonstrated elegantly in ERalpha-knockout mice, in which estrogens fail to exert a uterotrophic effect.[63] As would be expected, soy isoflavones have shown no impact on endometrial proliferation in clinical studies.[64-68] Although endometrium expresses both alpha and beta receptors, synthetic agonists specific for ERbeta do not decrease uterine weight in rats or prevent the proliferative response to a concurrently administered ERalpha-specific agonist.[36] In women, soy isoflavones do not suppress the endometrial proliferative response to estrogen.[69] Based on these observations, ingestion of genistein within the nutritional range would not be expected to either increase or decrease endometrial cancer risk.

Nonetheless, when rats are fed doses of genistein that could be considered pharmacological – for example, 750 mg/kg diet, resulting in a free serum genistein level of 400 nM – uterotrophic activity is indeed seen.[70] This finding is consistent with the responsiveness of ERalpha to high nanomolar concentrations of genistein. The implication is that genistein doses which greatly exceed the nutritional range should not be presumed to be safe from the standpoint of endometrial cancer risk.

**Genistein Up-Regulates eNOS Activity in Vascular Endothelium**

One of the chief reasons for suspecting that hormone replacement therapy would decrease cardiovascular risk is that estrogens have a favorable impact on endothelial function, promoting the activity of the endothelial isoform of nitric oxide synthase (eNOS); this results both from increased transcription, and also from extranuclear effects of activated extrogen receptors (both ERalpha and ERbeta) exerted at the plasma membrane.[71-76] Estrogen can also enhance the bioactivity of nitric oxide by down-regulating NADPH oxidase expression – more specifically, that of its gp91phox subunit - in human endothelial cells.[77] Vascular endothelium expresses both ERalpha and ERbeta, and their effects on human umbilical vein endothelial cells appear to be quite comparable.[78] Studies point to a role for ERalpha in the induction of eNOS and/or NO production in the endothelial cells of diverse species;[79-84] the impact of ERbeta in this regard is less clear.

Whereas estrogen increased the expression of eNOS in the coronary arteries of ovariectomized ERalpha knockout mice,[85] consistent with a role for ERbeta in eNOS induction, no such effect was seen in their cerebrovascular arteries.[81] Induction of eNOS by ERbeta in rat cardiac myocytes and in human myometrium has been reported.[86;87] In the vascular smooth muscle cells of rodents, ERbeta exerts both antihyperplastic and antihypertensive effects.[88;89]

Clinical observations appear consistent with the possibility that ERbeta supports endothelial eNOS activity in at least some vascular beds. Adequate oral intakes of genistein have been reported to improve endothelium-dependent vasodilation – as well as other markers for endothelial NO production - in postmenopausal women.[90-93] The most striking findings in this regard have been reported by Squadrito and colleagues, who administered either 54 mg free genistein daily, a typical oral hormone replacement regimen, or a placebo, to 90 postmenopausal women for 1 year.[91] Genistein treatment increased brachial endothelium-dependent vasodilation and post-occlusive blood flow relative to placebo; the effects of the hormone replacement regimen were quite similar. Moreover, plasma levels of nitric oxide metabolites were doubled, and plasma levels of endothelin-1 virtually halved, in both the genistein and hormone replacement groups. (This suppression of endothelin-1 may reflect the ability of NO to inhibit endothelial secretion of endothelin.)[94] Other studies have likewise reported favorable effects of soy isoflavone supplementation (or administration of isoflavone-rich soy protein) on vascular endothelial function in women – albeit a few studies have failed to observe such an effect.[95;96] One study reported an improvement in arterial compliance, but not in acetylcholine-mediated vasodilation.[97] In ovariectomized rats, dietary genistein has been shown to improve endothelium-dependent, but not endothelium-independent, vasodilation.[98-100]

Squadrito suggests that lower intakes of genistein, or shorter duration of supplementation, might account for the two negative reports. Indeed, each of these studies administered 80 mg of mixed conjugated soy isoflavones daily;[95;96] it would take about 150 mg of such a preparation to provide the 54 mg of pure genistein used in the Squadrito study. The fact that Squadrito administered free genistein, rather than the genistein glycoside (genistin) that occurs natively in unfermented soy foods, might also have some bearing in this regard. There are conflicting reports regarding the relative bioavailabilities of free isoflavones and conjugated isoflavones; one Japanese study concluded that, during longterm administration, administration of free isoflavones yielded plasma concentrations of total isoflavones that were roughly twice as high as those achieved during administration of conjugated isoflavones.[101] On the other hand, three recent American and Swiss studies conclude that, after single oral doses, the availabilities of the two forms of isoflavones are roughly comparable.[102-104] Although isoflavone glycosides are not taken up by intestinal cells,[105] these compounds are readily converted to free glycosides by membrane-bound or bacterial β-glucosidases in the intestinal tract;[106] the resulting free isoflavones are absorbable.[107] The propensity of gut bacteria to degrade isoflavones varies from person to person and influences their bioavailability – slow metabolizers achieve higher plasma levels of genistein and daidzein.[108] Since free isoflavones should be absorbed more rapidly, one would suspect that they would be more effective than

conjugates in rapid metabolizers – but this has not been documented. Evidently, more research is needed to evaluate the relative impact of free vs. conjugated isoflavones on plasma isoflavone levels during long-term administration.

The favorable effects of genistein on endothelial function might be operative in men as well, in light of a report that intrabrachial administration of genistein led to a nitric oxide-mediated increase in forearm blood flow in male volunteers; infusion of 17beta-estradiol – but not daidzein – had a comparable effect.[109] On the other hand, one study reported a modest reduction in endothelium-dependent vasodilation after men had ingested isoflavone-rich soy protein (40 g daily, providing 118 mg isoflavones) for three months.[110] The impact of dietary genistein on endothelial function in males should be studied further. Estrogen can boost endothelium-dependent vasodilation in males,[111-113] and studies in aromatase knockout male mice indicate that endogenous estrogen improves endothelial function in the males of this species.[114]

In light of the versatile role which endothelial nitric oxide production plays in preservation of vascular health, it seems likely that genistein's ability to up-regulate eNOS function could be exploited to decrease vascular risk. Epidemiological evidence suggests that estrogen may be largely responsible for the relative protection from cardiovascular disease enjoyed by premenopausal women.[115-117] It would be a fortunate development indeed if men could use genistein to achieve at least a portion of this benefit without incurring typical estrogenic side effects.

Sadly, induction of eNOS is not always an unalloyed benefit; in endotheliopathies associated with increased superoxide production, tetrahydrobiopterin deficiency leads to an "uncoupling" of eNOS that impairs its activity while turning it into a superoxide generator.[118-120] Fortunately, there is recent evidence that high-dose folic acid can restore the proper protective function of eNOS when tetrahydrobiopterin is deficient.[121-125] Thus, it is reasonable to suggest that concurrent administration of high-dose folate could be a prudent adjuvant to genistein supplementation in patients at risk for endothelial dysfunction. Supplemental arginine, as well as various measures which lessen superoxide production by NADPH oxidase, may also help to optimize the bioefficacy of the eNOS expressed by dysfunctional endothelium.[125-127]

**Favorable Effects on Bone Metabolism**

Both ERalpha and ERbeta are expressed in the main cell types present in human bone: osteoblasts, osteoclasts, and osteocytes.[128-130] Studies with osteoblast-derived cell lines indicate that these two receptors modulate the transcription of distinctly different sets of genes, with only a modest amount of overlap.[131] The effects of ERalpha on gene expression tend to be amplified in ERbeta knockout mice, suggesting that Erbeta down-regulates some responses to ERalpha.[132] Cortical bone density is greater, and age-related loss of trabecular density is lower, in ERbeta knockout mice as compared to wild type.[133] Yet in ovariectomized ERalpha knockout mice, estrogen promotes increased bone density – albeit not as effectively as it does in ERalpha knockouts; evidently, both types of estrogen receptor can act to preserve bone integrity.[134] Indeed, trabecular bone

density tends to be increased in ERalpha knockout female mice.[135] These effects may be sex-specific, as estrogen did not improve bone density in orchidectomized ERalpha knockout mice.[134] In a human osteoblast-derived cell line (MG63) in which ERalpha had been silenced with antisense plasmids, estrogen increased collagen and alkaline phosphatase secretion, demonstrating an anabolic effect of ERbeta in these cells.[136]

The increase in bone osteoclastic activity and bone resorption ushered in by menopause is thought to stem primarily from an alteration of osteoblast function; soluble mediators produced by osteoblasts have a major impact on the functional status of nearby osteoclasts. Estrogen inhibits osteoblast production of IL-6, an important trophic factor for osteoclasts; it also boosts production of osteoprotegerin, a soluble "false receptor" which inhibits activity of RANKL, another important trophic factor for osteoclasts.[14;137;138] It is thus of particular interest that, in physiological concentrations, genistein has been shown to suppress IL-6 production, and boost osteoprotegerin production, in human osteoblast-derived cell lines.[139;140] These effects are inhibited by concurrent incubation with an estrogen receptor antagonist. These findings strongly suggest that genistein can interact with ERbeta to achieve transrepression of the IL-6 promoter – as it does with the TNF promoter.[7]

Genistein also has the potential to improve bone metabolism through its impact on vascular eNOS, in light of recent evidence that this enzyme is a mediator of the osteogenic impact of estrogen on osteoblasts.[141-143]

In light of these considerations, it is not surprising that, in non-uterotrophic doses, genistein has repeatedly been shown to promote maintenance of bone density in ovariectomized rats and mice.[144-149] Whether this effect would be lost in ERbeta knockout mice – thus confirming that ERbeta mediates the effect – has not been determined. Surprisingly, two drugs said to be potent and selective ERbeta agonists did not inhibit bone loss in ovariectomized rats.[36;150] However, the fact that these agents have agonist activity for transcriptional promotion in some contexts, does not necessarily imply that they will have such activity in other contexts – nor that they will interact with ERbeta in a manner that achieves transrepression of the IL-6 promoter.

Clinical studies of supplementation with soy isoflavones or isoflavone-rich soy protein in postmenopausal women have reached divergent conclusions: some find that such supplementation has a favorable effect on markers of bone metabolism and on preservation of bone density,[26;151-156] whereas others do not.[157-159] Perhaps the most impressive study in this regard was that of Squadrito and colleagues.[26] These researchers recruited 90 postmenopausal women, 47-57 years of age, who were randomized to receive either genistein (54 mg daily), a standard HRT combination (17β-estradiol/norethisterone acetate), or a matching placebo; response was evaluated at 6 and 12 months. Genistein treatment was found to decrease excretion of pyridinium cross-links (a marker for collagen catabolism in bone) at both 6 and 12 months; this response was quite comparable to that achieved with HRT. In contrast to HRT, however, genistein increased serum levels of bone alkaline phosphatase and osteocalcin, markers for osteoblastic activity. At 12 months, the serum RANKL/osteoprotegerin ratio was notably

reduced in the genistein group, to a greater extent than in the HRT group.[156] Finally, after 12 months, bone mineral density in the femoral neck and lumbar spine had increased by 3-4% in both the genistein and HRT group, as contrasted to modest losses of density noted in the placebo group. This study is notable for its comparatively long duration – sufficient to evaluate changes in bone density – and for its use of a fairly ample dose of free genistein. The total serum genistein (conjugated plus free) in the genistein-supplemented group, measured during a morning fast, was 1.5 μM at 6 months and 1.7 μM at 12 months; assuming that about 1.3% of the genistein pool was unconjugated, this would be expected to correspond to a free genistein concentration of about 20 nM, presumably sufficient to activate ERbeta.

Most other studies of this type have administered isoflavones as glycosides, often in conjunction with soy protein. It is conceivable that variations in the bioavailabililty of genistin are a least partially responsible for the inconsistent findings of these studies. In future, such studies should measure serum free genistein levels to assess the bioavailability of the administered isoflavones. Isoflavone supplementation has also been assessed in premenopausal women and in men; no impact on bone metabolism or bone density was noted in these studies.[160;161]

Since East Asian women often consume ample amounts of soy isoflavones in their habitual diets, several studies have attempted to correlate habitual isoflavone intake or serum isoflavone level with postmenopausal bone density and/or bone metabolism in such women. Two studies – one each from Japan and China - have reported that women in the highest quartile of soy intake, as contrasted to the lowest quartile, had higher bone density.[162;163] Several other studies, including those in the U.S. or Europe, where soy intake is comparatively low, did not observe such a correlation, [164-166] albeit a Southern California study had a positive outcome.[167]

**Reducing Prostate Cancer Risk**

Human prostatic epithelium expresses ERbeta, but not ERalpha – whereas prostate stroma expresses ERalpha.[168;169] There is reason to believe that ERbeta activity has an antiproliferative impact both in healthy prostate and in prostate cancers.[170;171] Prostatic hypertrophy is common in aging ERbeta-knockout mice, whereas knockout of ERalpha has no such effect.[172;173] Furthermore, transfection of ERbeta into human prostate cancer cell lines induces apoptosis.[174] As prostate cancers progress, ERbeta expression tends to decrease – consistent with the possibility that this receptor exerts a restraining effect on proliferation.[169;175;176] In prostate cancer cell lines which express ERbeta, a variety of estrogens and anti-estrogens – including genistein and the drug raloxifene – have an antiproliferative, pro-apoptotic effect.[177-180]

Genistein has been shown to decrease expression of the androgen receptor in the human prostate cancer-derived LNCaP cell line, an effect mediated by activation of ERbeta.[181] Moreover, soy phytochemical concentrates slow the growth of LNCaP in nude mice.[180;182;183] Genistein feeding likewise down-regulates androgen receptor expression in rat prostate,[184] and reduces the yield of prostate cancer in carcinogen-treated rats as

well as in transgenic "TRAMP" mice that have a high spontaneous incidence of this cancer.[185;186] Pilot clinical studies evaluating the impact of oral genistein on early stage prostate cancer have achieved a moderate reduction of PSA in a minority of patients, and an apparent reduction in cancer growth rate in others.[187;188] Case-control studies from the Orient are reasonably consistent with the thesis that diets high in soy products are associated with lower risk for prostate cancer[189-191] – albeit high soy intake may be a marker for diets and lifestyles that are more traditional.

**Isoflavones and the Breast – Safe and Possibly Protective**

How dietary soy isoflavones influence breast cancer risk is also a matter of considerable interest. In the normal human breast, both types of estrogen receptor are expressed in epithelial cells;[192] ERbeta predominates in adult human mammary fibroblasts.[193] In ERalpha knockout mice, the breast is atrophic; conversely, in ERbeta knockout mice, epithelium is hyperproliferative and the mice are prone to severe cystic breast disease as they age.[194;195] Transfection of ERbeta into an ERalpha-expressing human breast cancer cell line (MCF-7) results in a suppression of proliferation associated with up-regulation of cdk inhibitors p21 and p27, and down-regulation of c-myc and cyclins D1 and A; however, these effects are only partially ligand dependent.[196] ERbeta transfection also slows the estrogen-stimulated growth of the estrogen-sensitive T47D mammary cancer cell line,[197] but has a pro-proliferative impact on the MDA-MB-435 tumor.[198] In the main, these findings suggest that ERalpha and ERbeta may have a "yin-yang" role in breast development, with ERbeta opposing the proliferative impact of ERalpha; however, they do not necessarily imply that ERbeta-specific ligands will have an antiproliferative effect.

ERbeta is expressed by the majority of human breast cancers – even those considered "estrogen negative";[199;200] the standard assays for breast cancer "estrogen receptors" are ERalpha-specific. The prognostic significance of ERbeta expression in breast cancer has been the subject of conflicting reports.[196]

A number of studies have examined the impact of soy isoflavones on breast cancer risk or growth in rodents, but some of these are of limited interest owing to their use of high parenteral doses that would likely have ERalpha-agonist activity. High-dose genistein, administered pre-pubertally, induces a premature differentiation of breast tissue that diminishes susceptibility of adult rats to carcinogen-induced breast cancer;[201-203] this effect is also seen with estrogen administration, and there is no evidence that physiological levels of genistein could achieve a comparable effect. There are however several studies which conclude that more modest intakes of genistein can favorably influence breast cancer induction. When administered at 250 mg/kg diet, either genistein or daidzein was found to slow the onset of breast cancer in cancer-prone MMTV-neu mice; however, they did not influence the growth of established tumors.[204] A functional food rich in unconjugated genistein slowed the growth of the MDA-MB-231 human breast cancer in nude mice; this was associated with increased apoptosis in the tumor.[205] In the mouse mammary tumor virus-induced spontaneous breast cancer model, oral administration of biochanin A – but not daidzein – reduced tumor incidence at 15

months; this effect was not seen in germ-free animals, and thus presumably was contingent on conversion of biochanin A to genistein by intestinal bacteria.[206]

On the other hand, a number of studies show that dietary genistein can increase the growth of estrogen-dependent MCF-7 tumors in ovariectomized nude mice; this effect appears to hinge on activation of ERalpha.[207-210] This cell line seems to be exquisitely sensitive to ERalpha activation, inasmuch as genistein concentrations as low as 10 nM can modestly increase its rate of proliferation in vitro.[207;211] Nonetheless, a much more substantial response is seen with genistein concentrations in the high nanomolar range, consistent with the known affinity of genistein for ERalpha; responsiveness at 10 nM presumably reflects that fact that activation of only a small minority of ERalpha receptors can have a discernible impact on proliferation in this cell line. Administered at 750 mg/kg of diet to rats pretreated with the carcinogen MNU, genistein increases the size of MNU-induced mammary tumors in ovariectomized rats;[212] this dose of genistein also increases uterus size, pointing to an ERalpha-mediated effect on estrogen-sensitive tumors. In DMBA-treated mice, 1 g/kg dietary genistein increases the yield of malignant adenocarcinomas – whereas no cancers develop with ERalpha knockout mice.[213] Overall, these findings suggest that moderate intakes of genistein may slow the onset or progression of certain mammary tumors, but that very high intakes can be expected to boost the growth of estrogen-dependent tumors, and even moderate intakes may have the potential to at least modestly influence the growth of certain tumors that are highly sensitive to ERalpha activation.

The impact of soyfood ingestion on breast cancer incidence has been examined in a number of case-control as well as prospective epidemiological studies. Although many of these studies find no link between breast cancer and soy consumption [214-218], four case-control studies found an inverse association between soy intake and risk for premenopausal breast cancer in Asian populations, and two such studies found a similar association with postmenopausal breast cancer.[219-223] One recent prospective Japanese study found that high intakes of miso or of isoflavones – but not of soyfoods per se – were associated with decreased risk for pre- or postmenopausal breast cancer.[224] Other prospective studies have had a negative outcome. One case-control study in Asian-Americans focused on soy consumption during adolescence, and found that high soy intake during this time predicted a lower risk for postmenopausal breast cancer; such risk was lowest for those who maintained high soy consumption in adult life.[223] Given the fact that most Asian diets provide suboptimal isoflavone intakes from the standpoint of ERbeta activation, these findings are reasonably consistent with the possibility that somewhat higher supplemental intakes of genistein might be protective in regard to breast cancer risk; however, they certainly don't prove this proposition. A conservative but optimistic perspective is that, as contrasted with HRT, there is little reason to suspect that genistein intakes in the high *nutritional* range would *increase* breast cancer risk – and some reason to suspect that such a measure might decrease this risk.

On the other hand, in women who have been diagnosed with estrogen-sensitive breast cancers, the possibility cannot be excluded that nutritional intakes of genistein will modestly boost cancer growth by promoting low-level activation of ERalpha.

Theoretically, selective activation of ERbeta with moderate-dose genistein might slow the growth of certain estrogen-sensitive mammary cancers which express this receptor - but this is speculative. Until further evidence is forthcoming, it might be prudent for women with estrogen-sensitive breast cancer to refrain from frequent soy ingestion or isoflavone supplementation.

**Modestly Effective for Hot Flashes**

A number of clinical studies have assessed the impact of supplemental soy isoflavones – with or without soy protein – on postmenopausal hot flashes. A recent overview notes that 4 of these studies had a positive outcome, 5 were negative, and one showed a positive trend that missed statistical significance.[225] This overview could not include a more recent study by Squadrito and colleagues.[226] These researchers nested a hot flash study into their bone metabolism study by enrolling only women who were troubled by this complication; since this study achieved free genistein concentrations sufficient to benefit bone metabolism, and since it included an HRT arm, its findings may be particularly illuminating. At baseline, the daily hot flash score was very similar in the three groups, averaging 4.5-4.7 per day. After 3 months, as contrasted with the placebo group, this score was 22% lower in the genistein group and 53% lower in the HRT group; these differences were statistically significant. The findings at 12 months were similar – relative to placebo response, the score was 24% lower with genistein and 54% lower with HRT. Response to HRT was significantly greater than that to genistein.

The available findings appear consistent with the proposition that soy isoflavone regimens which achieve an adequate plasma level of free genistein are mildly beneficial with respect to hot flashes – though less effective in this regard than HRT. The inconsistency of the results of clinical studies examining this issue, may reflect the fact that the benefit to be expected is modest, as well as the likelihood that some of the isoflavone regimens tested failed to achieve adequate free genistein concentrations in some subjects. It seems highly unlikely that about half of the clinical studies to date examining this issue would find statistically significant benefit, if in fact isoflavones had no genuine potential for controlling hot flashes. Soy isoflavones have access to the brain, and certain regions of the hypothalamus express ERbeta;[227-229] conceivably, some of these receptors mediate the impact of genistein on hot flashes.

**Potential for Prevention of Glomerulosclerosis and Left Ventricular Hypertrophy**

Chronic renal disease, of either diabetic or non-diabetic origin, tends to progress less rapidly in women than in men.[230-232] The relative protection enjoyed by women appears to be confined to the premenopausal period, and thus is likely to be mediated by estrogen.[232;233] Indeed, estrogen ameliorates, whereas ovariectomy exacerbates, the progression of glomeruloslerosis in various rodent models of this disorder.[234-240] Contrary findings have been reported in certain hyperlipidemic strains of rodents, presumably because estrogen treatment exacerbates nephrotoxic hyperlipidemia in these animals;[241-244] an estrogen-evoked increase in growth hormone secretion can also exert a

countervailing negative effect in this regard.[245-247] However, these latter findings do not appear to be germane to the impact of endogenously-produced estrogen in women.

The various agents and circumstances which provoke glomerulosclerosis – such as hyperglycemia, glomerular hypertension, angiotensin II, advanced glycation endproducts, thromboxane, and oxidized LDL – appear to do so by boosting glomerular production of transforming growth factor-beta (TGF-β); activation of AP-1 response elements in the TGF-β promoter, often in response to PKC/MAP kinase activation, plays a role in these inductions.[248-261] This increased autocrine/paracrine production of TGF-β, in turn, activates mesangial receptors for this hormone, leading to increased production of various ground substance proteins – including collagen types I and IV, laminin, and fibronectin – decreased production of the collagenases MMP-2 and MMP-9, and increased production of protease inhibitors such as TIMP-1;[262-272] the net effect is an accumulation of mesangial ground substance and a thickening of glomerular basement membranes. TGF-beta is also a mediator of the proteinuria characteristically seen in glomerular disorders.[273-277] Injection of anti-TGF-β antibodies into diabetic rodents can prevent and in some measure reverse glomerulosclerosis – demonstrating the central role of TGF-β in this syndrome.[277-280]

Mesangial cells express both ERalpha and ERbeta;[232;281] it is noteworthy that expression of both types of ER is diminished in this mesangial cells of a strain of mouse prone to glomerulosclerosis.[232] Physiological concentrations of estrogen have been shown to suppress the response of mesangial cells to TGF-β.[282;283] This effect appears to reflect the ability of activated estrogen receptors of both types to bind to, and suppress the transactivating activity of, the transcription factor SMAD3,[284] whose phosphorylation and activation by TGF-β receptors mediates most effects of TGF-β.[285] (Not surprisingly, SMAD3-knockout mice are virtually immune to diabetic glomerulopathy.)[276] Moreover, estrogen can also suppress glomerular production of TGF-β, by boosting the NO production of glomerular endothelial cells.[286] NO's suppressive impact on TGF-β production is poorly understood; the fact that eNOS inhibitors up-regulate glomerular TGF-β synthesis suggests that this mechanism is of physiological significance. It does not appear to be known whether glomerular endothelial cells express ERbeta, or whether this receptor can enhance eNOS activity in these cells. A further theoretical possibility is that activated ERbeta could inhibit transcription of TGF-β by interfering with AP-1 activity in its promoter; on the other hand, ERalpha would be expected to enhance AP-1 activity.[15;20] In any case, there is reason to suspect that high-physiological concentrations of genistein, via activation of ERbeta, could inhibit the effects of TGF-β on mesangial cells, thereby helping to prevent glomerulosclerosis. Whether activation of ERbeta could also increase glomerular NO production and/or interfere with AP-1 activity – resulting in suppression of glomerular TGF-β production – is more speculative.

In fact, many rodent studies conclude that soy-based diets – in comparison to casein-based diets – are associated with slower progression of glomerulosclerosis.[287-295] Two recent clinical studies in type 2 diabetics likewise report that proteinuria is lower during soy protein supplementation than during casein supplementation.[296;297] At least a portion of this effect reflects the fact that soy is of "poorer quality" than casein. It is well

established that diets rich in "high quality" protein promote glomerulosclerosis by increasing glomerular pressure and thereby increasing glomerular filtration rate; this presumably represents a homeostatic response which helps the body to cope with increased nitrogenous waste. [291;298;299] Soy and other plant proteins have a less substantial effect in this regard.[300-302] Thus, diets low but adequate in protein content have been used clinically to slow the progression of glomerular disease;[303;304] in particular, quasi-vegan diets, some featuring soy protein, have been used for this purpose.[301;305-308]

The possibility that phytochemical components of soy – such as isoflavones – contribute to the nephroprotection afforded by soy-based diets has been suggested by Valasquez and Bhathena.[291;309] Indeed, a subsequent study with hypercholesterolemic rats prone to glomerulosclerosis demonstrated that addition of an isoflavone-rich soy ethanol extract to their casein-based diets ameliorated subsequent renal damage; the interpretation of this study is complicated by the fact that serum lipids declined in the isoflavone-supplemented rats.[310] Also of particular interest in this regard is a study by Neugarten and colleagues.[311] These researchers demonstrated that, in concentrations as low as 1-10 nM, genistein markedly inhibits synthesis of both type I and type IV collagen by murine mesangial cells; the authors propose that ERbeta mediates this effect. Conceivably, this finding simply reflects the fact that genistein-activated ERbeta can block the impact of autocrine TGF-β on mesangial cells.[284] Whether physiological concentrations of genistein can also influence glomerular production of TGF-β remains to be seen. Of related interest is a report that a diet supplemented with red clover isoflavones decreases production of TGF-β by prostatic epithelium in mice.[312]

If subsequent animal and clinical studies prove that soy isoflavones can indeed reduce risk for, or slow progression of, glomerulosclerosis, it won't necessarily follow that a diet high in soy protein should be recommended. Most likely, the most nephroprotective diet will be a vegan diet relatively low in protein content, supplemented with soy isoflavones. In this regard, adding soy protein to a diet already rich in casein did not protect hypercholesterolemic rats from glomerular injury – albeit addition of a comparable surplus of casein exacerbated this injury.[293]

The intracellular signaling mechanisms which mediate left ventricular hypertrophy (LVH) appear to be very similar to those that evoke glomerulosclerosis;[313;314] moreover, NO has an antagonistic impact on development of LVH analogous to its impact on glomerulosclerosis.[315] Premenopausal women are relatively protected from LVH, and estrogen replacement has been shown to limit expansion of ventricular mass in postmenopausal women and ovariectomized rodents at risk for this disorder.[316-323] Cardiac myocytes and fibroblasts express both isoforms of the estrogen receptor, but, in neonatal rat cardiac myocytes, only ERbeta has an inductive effect on eNOS.[324;325] A very recent study examining ERalpha- and ERbeta-knockout mice demonstrates that ERbeta mediates the protective effect of estrogen on cardiac hypertrophy.[326] Thus, it is reasonable to suspect that genistein has the potential to protect postmenopausal women, and possibly men, from LVH.

**Directions for Future Research**

The thesis that ERbeta mediates the favorable physiological effects of moderate-dose isoflavones on bone metabolism and endothelial function can best be tested in ovariectomized ERbeta knockout rodents, using dietary concentrations of isoflavones that will achieve a free genistein plasma concentration not in excess of 50 nM. If these effects largely persist in the ERbeta knockouts, the thesis of this paper will be falsified, and it will be necessary to identify further molecular targets that respond to free isoflavones in the low nanomolar range. In light of current evidence, however, the contention that ERbeta is the key target of dietary isoflavones is credible and brings a satisfying unity to the diverse research literature on these compounds.

The fact that daidzein has a relatively low affinity for both ERbeta and ERalpha, may seem difficult to square with a handful of reports indicating that daidzein, or its methoxylated derivative formononetin, can be physiologically active.[327-330] GI bacterial action has the potential to convert these compounds to equol, the S-isomer of which has recently been shown to be a potent and selective agonist for ERbeta, with an affinity almost as high as that of genistein. [331;332] The R-isomer, on the other hand, is relatively selective for ERalpha, and has a higher affinity for this receptor than does genistein ($K_i$ = 50 nM). Too little is currently known about the extent of glucuronidation of plasma equol, or the relative abundance of the two isoforms, to make firm predictions regarding the possible contribution of equol to the physiological effects of soy isoflavones. Further complicating this issue is the fact that capacity to convert daidzein to equol varies a great deal from person to person, to the extent that people have been categorized as equol "producers" or "non-producers".[331;333] Since soy isoflavone supplementation, at least in nutritional doses, is non-uterotrophic, it is reasonable to conclude that R-equol makes little contribution to the physiological effects of such supplementation. Whether S-equol has a more significant physiological role – thus rationalizing claims that daidzein or formononetin are clinically active – must be clarified in future research.

The fact that clinical studies with soy isoflavone supplementation have yielded inconsistent results may reflect, at least in part, variations in the plasma levels of free genistein (and possibly equol) achieved by the diverse supplementation regimens that have been assessed. Is genistin truly as effective as equimolar intakes of genistein during longterm administration in most subjects? Or do variations in GI metabolism of genistin render supplementation with genistein a more fail-safe proposition? Too few studies have assessed the long-term impact of various isoflavone regimens on equilibrium concentrations of free genistein; we should bear in mind that acute pharmacokinetic studies do not take into account possible adaptive changes in enzyme expression that could influence achieved plasma levels. In light of the markedly beneficial effects on endothelial function and bone metabolism achieved by Squadrito et al. with 54 mg genistein daily, efforts to confirm these results in larger and more diverse populations are clearly warranted.

As a safer substitute for HRT in postmenopausal women, supplemental genistein would appear to have great promise; in Squadrito's studies, the impact of supplemental genistein

on endothelium and bone was fully comparable to that of HRT, and the relief from hot flashes noted was worthwhile though less substantial. Whether premenopausal women would benefit is less clear, since the impact of genistein on endothelium or bone may be modest compared to that of ambient estrogen levels. However, the epidemiological and rodent literature provides just a hint that, by shifting the balance toward ERbeta activity in mammary tissue, premenopausal soy isoflavone ingestion may in fact be protective in regard to breast cancer risk. In this regard, more rodent chemoprevention studies, using moderate genistein doses that will selectively activate ERbeta, would be desirable; in regard to epidemiology, more attention should be focused on the possible impact of adolescent isoflavone intake on subsequent breast cancer risk.

Could supplemental genistein improve endothelial function and bone metabolism in men? Too few studies have examined this issue to allow any conclusions to be drawn. However, even if genistein cannot protect men in these respects, its likely impact on prostate health may make genistein supplementation a very worthwhile option for men. Ongoing clinical studies with genistein in early prostate cancer may shed further light on this issue. The fact that ERbeta expression tends to be lost as prostate cancer progresses probably means that, as a therapy for pre-existing prostate cancer, genistein will have at best transient efficacy; thus, its greater potential may be for chemoprevention.

With respect to expectations that isoflavone-rich diets may decrease risk for certain common cancers, a proviso is in order. Commentators frequently note that risks for certain "Western" cancers are comparatively low in East Asian cultures which make frequent use of soy products. However, these rates are equally low in many other Third World rural societies in Africa and South America where soy consumption is minimal.[334-336] The traditional diets of these societies tend to be low in fat and animal products, and moderate in total protein; lifelong consumption of such diets is likely to be associated with reduced serum levels of insulin and of free IGF-I – now known to have important cancer promotional activity.[337-339] High intakes of soy protein can actually boost serum IGF-I;[340-342] thus, heavy use of soy protein per se may be inadvisable from the standpoint of cancer risk. These considerations suggest that ample intakes of soy isoflavones may best be achieved through supplementation rather than through heavy consumption of protein-rich soy products. Furthermore, we should take care not to encourage the delusion that simply adding soy products – or soy isoflavones – to a meat-rich omnivore diet will reproduce the full measure of cancer protection associated with quasi-vegan Third World diets. The popular focus on soy protein runs the risk of obscuring the broader and deeper truth that diets featuring "low quality" plant protein can have an important anti-promotional impact on many types of cancer.[339;343;344]

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