Phytoestrogens

K S Sebastian and Raghava Varman Thampan*
Rajiv Gandhi Centre for Biotechnology, Thiruvanathapuram 695 014, India
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Phytoestrogens are chemical agents, originated from plants, which display structural and functional similarity to 17β estradiol and show both estrogen agonist and antagonistic properties. During recent years phytoestrogens attracted immense public attention as an alternative to estradiol in estrogen replacement therapy for treating postmenopausal women. Epidemiological data have shown a strong correlation between the incidence of hormone dependent diseases and the nature of the diet. Different phytoestrogens exert their biological effects through different mechanisms and through different types of receptors, the expression of which appears to be tissue specific. Though there is considerable amount of epidemiological data indicating the possible health benefits of phytoestrogens, for better utilization of these agents a deeper understanding of the mechanism of action of these estrogens is essential. This will help in the identification of a particular type of phytoestrogen, which can elicit a tissue specific effect under a given clinical condition.

Keywords: phytoestrogens, estrogen receptor α, non-activated estrogen receptor, nuclear estrogen receptor II, estrogen receptor activation factor

Introduction

Estrogenic activity was first reported in plant extracts way back in 1927, which was a natural outcome of the publication of the Allen Doisy bioassay for estrogens. The importance of phytoestrogens was recognized initially following the discovery of cases of infertility in sheep grazing on pastures rich in subterranean clover. This phenomenon has been identified as the ‘Clover Disease’ (Murkies et al, 1998). More recently, their importance became additionally pronounced in view of the potential anticancer properties associated with these agents (Adlercreutz et al, 1993; Castle & Thrasher, 2002; Gantry, 2002). Other potential beneficial effects of phytoestrogens include the protection they provide against cardiovascular diseases (Yvone et al, 2002; Herrington, 2000; Tikkanen & Adlercreuz, 2000; Lissin & Cooke, 2000), brain dysfunction (Linford & Dorsa, 2002; Gelinis & Martinoli, 2002), bone resorption (Ewies, 2002; Jie Mei et al, 2001; Draper et al, 1997), obesity and diabetes (Bhathena & Velasquez, 2002) and also against renal diseases (Velasquez & Bhathena, 2001). A negative report that phytoestrogen stimulates the proliferation of breast cancer cells in vitro has also been published (Welshons, 1987). This is a factor that needs to be given serious attention prior to testing a given phytoestrogen in a clinical study. Phytoestrogens are gaining importance as an alternative to natural estrogens in hormone replacement therapy (Russell et al, 2002) as there is increasing evidence of their overwhelming health benefits.

Phytoestrogens Currently Known and Their Sources

The major classes of phytoestrogens that have been identified so far are coumestans, isoflavonoids, flavonoids, lignans and mycoestrogens (Belcher & Zsarnovszky, 2001). Isoflavonoids includes isoflavones, (formononetin, daidzein, biochanin A, genistein and ipriflavone) isoflavanones (o-desmethylangolensin) and isoflavans (Equol). Flavonoid group comprises of flavones, (apigenin, chrysin, and flavone) flavanones (Naringenin). Coumestans group is represented by coumestrol, and lignans category includes enterolactone and enterodiol. Important mycoestrogens are zearalenone, α-zearalenol, and β-zearalenol (Belcher & Zsarnovszky, 2001). In a human dietary exposure perspective, the significant groups are isoflavonoids, coumestans and lignans. Isoflavones, genistein and daidzein are also present in a wide variety of fruits and nuts (Liggins et al, 2000). Coumestrol is the most potent among phytoestrogens. It is estimated to be
eral phytoestrogens fit very well into the available receptor can be attributed to the size of its hormone molecular volume of 17β-estradiol. Obviously several binding cavity, which has a volume twice that of the species. Obviously several phytoestrogens fit very well into the available space in the receptor. However, it is difficult to understand why other phytoestrogens do not exhibit higher binding affinities since the spatial orientation of the nonsteroidal ligands within the binding pocket remains largely unknown (Kuiper et al, 1998). The binding affinity of a given phytoestrogen need not necessarily be a factor that predicts its transactivation potential (Belcher & Zsarnovszky, 2001). In the case of isoflavones, the relative binding affinity of daidzein for ERα and ERβ is 1000 and 2000 fold lower, respectively than that of estradiol. However, the transactivation potential of daidzein (1000nM) at an estrogen responsive element was found to be nearly equivalent to that observed for the same concentration of estradiol (Kuiper et al, 1998). The relative binding affinity of genistein is 25 times lower than that of estradiol at ERα, whereas at ERβ the binding affinities of genistein and estradiol are similar. In contrast to the observed differences in the binding affinities of genistein and estradiol essentially, the transactivation activation function of 1000 nM genistein at ERα and ERβ is about 2 fold greater than a similarly high concentration of estradiol (Kuiper et al, 1998). The physiological relevance of the differences between estradiol and genistein in ER mediated transactivational function at the estrogen responsive elements remains uncertain since the estrogen concentration used were supraphysiological in nature (Belcher & Zsarnovszky, 2001). In contrast to estradiol, which effectively triggers both ERα and ERβ mediated transcriptional activation; isoflavones are weak estrogen agonists since they are effective only at triggering transcriptional activation or repression with ERβ and not with ERα. This difference can be explained by the estradiol dependent nonselective recruitment of co-regulators to ERα and ERβ, and the apparently selective recruitment by isoflavones. Genistein is very weak at recruiting glucocorticoid receptor interacting protein 1 (GRIP1) to ERα, but is active at recruiting GRIP1 to ERβ; thereby triggering ERβ mediated pathways (Jinping et al, 2001). It is unclear why genistein recruits GRIP1 more effectively to ERβ than to ERα. However, the binding of GRIP1 may stabilize the genistein-ERβ complex more effectively than the genistein-ERα complex because the binding of co regulator has been shown to slow the rate of dissociation of an agonist from ER-co-regulator complex (Gee et al, 1999). A list of the currently known phytoestrogens has been given in Table 1; also the molecular structure of some selected agents are being provided along with those of 17β-estradiol and diethylstilbestrol (Fig. 1).

**Possible Health Benefits**

Evaluation of the possible benefits and risks of phytoestrogens is not a simple process in view of the
interindividual diversity and complexity observed in the absorption and metabolism of the dietary phytoestrogens. However, results obtained from field studies clearly suggest that consumption of a diet rich in phytoestrogens is protective against breast, prostate and bowel cancer and also that it reduces estrogen deficiency symptoms in postmenopausal women. Consequently, there is a global movement and awareness towards increased consumption of food rich in phytoestrogens. This has resulted in the commercial production of concentrated isoflavone extracts as ready to use therapeutic agents (Davis et al, 1999).

**Estrogen and Cancer**

The incidence of hormone dependent tumours in the reproductive tract and accessory tissues is lower in women of Asia and Eastern Europe than those from the Western Europe and the US (Adlercreutz, 1990; Rose et al, 1986). This incidence is significantly low amongst vegetarians (Baghurst & Rohan, 1994; Hunter et al, 1996). A substantially reduced urinary excretion of total isoflavonoids and mammalian ligans was noticed in patients with cancer in contrast to the normal controls (Qi Dai et al, 2002). Animal studies using classical models of chemically induced breast cancer showed that a diet containing soy protein significantly reduced tumour formation (Troll et al, 1980; Hawrylewicz et al, 1991). This effect was neutralized when isoflavones were removed from the soy protein. *In vitro* studies using several different cell lines, including estrogen receptor independent cells (Peterson & Barnes, 1991) have confirmed the observations from *in vivo* studies regarding tumour suppression by isoflavones. What has become apparent here is that the mechanism of action of these phytoestrogens may not have an exclusive hormonal perspective (Barnes & Peterson, 1995; Barnes, 1995). Genistein is a specific inhibitor of protein tyrosine kinases (Akiyama et al, 1987) and topoisomerases (Okura et al, 1988; Markovits et al, 1989). It arrests cell growth by interfering with the signal transduction pathways (Tragonos et al, 1992; Matsukava et al, 1993; Higashi & Ogawara, 1994). The non activated estrogen receptor (naER) of the mammalian uterus is a tyrosine kinase and its exposure to estradiol leads to an inhibition in the enzyme activity. Whether genistein brings about a similar inhibitory effect on the tyrosine phosphorylation by naER remains to be known. Phytoestrogens exhibit many other properties like antioxidant (Tikkanen & Adlercreutz, 2000; Mizutani et al, 2000; Jha et al, 1985) and antiproliferative (Hirano et al, 1989; Yanagihara et al, 1993) activities and they inhibit the action of cytokines and growth factors (Higashi & Ogawara, 1994; Kim et al, 1998). Possibly, all these factors could either individually or collectively contribute to the potential anticancerous property of isoflavones. Phytoestrogens have the ability to inhibit sulphotransferases. These enzymes are known to be involved in the activation of some dietary procarcinogens (Kirk et al, 2001). Inverse correlation between soy product consumption on the one hand and colon tumour incidence on the other may be due to the enhanced synthesis of the antimitotic hormone, 1,25-dihydroxyvitamin-D3 under its influence (Kallay et al, 2002). Phytoestrogens also influence sex hormone metabolism by inducing the production of sex hormone binding globulin (SHBG) in the liver (Adlercreutz et al, 1986). They could also regulate estrogen metabolism by competing for the estrogen binding sites of the estradiol biosynthesizing and metabolizing enzymes, such as aromatase and

### Table 1—A list of currently known phytoestrogens

<table>
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<tr>
<th>Class</th>
<th>Compound</th>
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<tr>
<td>Coumestans</td>
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<td>BiochaninA</td>
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<td>Daidzein</td>
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<td>Formononetin</td>
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<td>Genistein</td>
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<td>Ipriflavone</td>
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<td>Isoflavanones</td>
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<td>O-Desmethylangolensin</td>
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<td>Isoflavans</td>
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<td>Equol</td>
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<td>Kaempferol</td>
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<td>Naringenin</td>
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<td>Lignans</td>
<td>Enterolactone</td>
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<td>Enterodiol</td>
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<td>Mycoestrogens</td>
<td>Zearalenone</td>
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<td>α-Zearalenol</td>
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<td>β-Zearalenol</td>
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Estrogen specific 17β hydroxysteroid oxidoreductase type I (Santii et al, 1998).

**Estrogenic Influence on the Bone**

The discovery of estrogen receptors in osteoblast cells (Komm et al, 1988; Eriksen et al, 1988) and the importance of estrogen in down regulating the activity of osteoclasts, thereby limiting bone resorption, have received wide recognition. Considerable evidence has accumulated suggesting that estrogen prevents bone loss by blocking the production of proinflammatory cytokines by bone marrow and the bone cells (Pacifici, 1996; Manolagas & Jilka, 1995). The main consequence of increased cytokine production in the bone microenvironment is an expansion of the osteoclastic pool due to increased osteoclast formation and elongation of their life span. Among the cytokines now known to be regulated by estrogens are IL-1, IL-6 and TNF (Pacifici, 1996; Manolagas & Jilka, 1995). IL-1 and TNF are the most powerful locally produced stimulators of bone resorption and are well recognized inhibitors of bone formation. IL-1 and TNF activate mature osteoclasts indirectly via a primary effect on osteoblasts and inhibit osteoclast apoptosis. In addition, they markedly enhance osteoclast formation by stimulating the proliferation of osteoclast precursors. This was achieved both directly and also by enhancing the pro-osteoclastogenetic activity of stromal cells. IL-1 and TNF are also powerful inducers of other cytokines that regulate the differentiation of osteoclast precursor cells into mature osteoclasts: such as IL-6, M-CSF and granulocyte M-CSF (Pacifici, 1998).

Phytoestrogens have the potential for minimizing bone loss in postmenopausal women. This has been demonstrated by the results of the clinical use of ipriflavone (Brandi, 1993). This was prescribed as an approved alteration to estradiol in estrogen replacement therapy for treating acute ovarian deficient states. The lack of regulation of ERα mediated genes and the potent repression of ERβ mediated genes by isoflavones may account for the low incidence of postmenopausal osteoporosis in Asian women (Ingram et al, 1997; Murkies et al, 1998; Tham et al, 1998). Studies conducted by Jinping et al (2001) suggests that ERβ mediated repression of the TNFα gene may be an important mechanism whereby isoflavones may prevent osteoporosis. Excessive production of TNFα after menopause is thought to lead to osteoporosis (Pacifici, 1996). Another suggested mechanism by which phytoestrogens could prevent bone resorption and bone loss is by enhanced osteoblastic production of osteoprotegerin (OPG) (Viereck et al, 2002). Receptor activator of nuclear factor kappaB ligand (RANKL) is the essential factor for osteoclast formation and activation, and enhances bone resorption. Osteoprotegerin (OPG), which is produced by osteoblastic lineage cells, serves to neutralize RANKL and, as a consequence prevents bone loss. 17β estradiol was found to stimulate OPG mRNA levels and protein secretion in a human osteoblastic cell line through activation of the ERα. Genistein increased OPG mRNA levels and protein secretion in human trabecular osteoblasts obtained from healthy donors. Simultaneous treatment with ER antagonist ICI 182780 completely abrogated the stimulatory effects of genistein on OPG protein secretion.
indicating that these effects of genistein were specific and directly mediated through the ER (Viereck et al., 2002).

Estrogen and Cholesterol Metabolism

Jinping et al (2001) hypothesized that ERβ selective estrogens such as isoflavones may prevent some postmenopausal symptoms and conditions. It has been observed that the helix12 of the AF-2 surface exists in a different position when genistein is bound to ERβ (Pike et al, 1999) than when estradiol is bound to the receptor (Brozozowski et al, 1997). Cholesterol homeostasis is an estrogen sensitive phenomenon. In the rhesus monkeys, studies using diets made from soy isolates (20%) with and without isoflavones showed that phytoestrogens contributed to a hypocholesterolemic effect. These studies showed relatively large reduction in total LDL and VLDL cholesterol in both male and female animals without any effects whatsoever on the reproductive tract (Setchell, 1998; and references therein). Isoflavones may slow the development of plaque formation by inhibiting cell adhesion and altering the activity of specific growth factors, such as platelet derived factor and cytokines, which influence lesion formation (Raines & Ross, 1995). Since oxidative modification of LDL is an important mechanism in atherosclerosis, it has been considered that the antioxidant properties of isoflavones could possibly reduce the extent of lipid peroxidation (Jha et al, 1985; Wei et al, 1995). The antioxidant activity of genistein is a factor that inhibits oxidative damage of calf thymus DNA induced by ultra violet light exposure in vivo (Wei et al, 1996). Genistein has also been found to prevent hepatic injury caused by the release of inflammatory cytokines during ischemia and reproduction (Yamamoto et al, 1996).

Adverse Effects

The most likely risks associated with phytoestrogen exposure deal with problems related to infertility and development. Phytoestrogens contained in dry summertime grasses have been reported to reduce the number of offsprings in wild populations of California quail (Leopold et al, 1976) and deer mice (Berger et al, 1977). Australian sheep have been shown to suffer from reproductive abnormalities and infertility after grazing in pastures with clover, Trifolium subterraneum that contained phytoestrogens. Rat pups exposed to high doses of the plant estrogen coumestrol through their mother’s milk, suffered permanent re-

productive problems: adult female rats failed to ovulate, while the males displayed altered mounting behaviour and fewer ejaculations (Whitten et al, 1993). Thigpen et al (2001) observed a marginal, yet significant inverse correlation between the total daidzein and genistein levels in circulation and the incidence of vulvar carcinoma in mice. Phytoestrogens may act as antiestrogens by competing for the binding sites of estrogen receptors or the active site of the estradiol biosynthezing and metabolizing enzymes such as aromatase (type I). In this instance these molecules could harm the reproductive health of males also (Santti et al, 1998). Yellayi et al (2002) reported thymic atrophy in mice following exposure to dietary genistein.

Target Sites for Phytoestrogen Action in Estrogen Responsive Cell

The various molecular targets for estrogen binding are given in Fig. 2. Emphasis has been given to the research work that has been accomplished at RGC laboratory, Thiruvananthapuram. The non activated estrogen receptor (naER) is a plasma membrane localized estrogen receptor that remains anchored to a plasma membrane protein in the absence of estradiol (Karthiskeyan & Thampan, 1996; Sreeja, 2002). Estradiol binding to naER leads to the receptor internalization through a clathrin coated vesicle (CCV) dependent mechanism. Following internalization its subsequent movement towards the nucleus (mediated by a 58 kDa NLS binding protein) does not appear to be an estrogen dependent process.

ERα moves into the nucleus with the help of a 55 kDa protein that recognizes the nuclear localization signals (NLS) on the receptor (Nirmala & Thampan, 1995 a & b). In the absence of estradiol bound to the receptor, ERα has its NLS masked by a 28 kDa protein (p28). Estradiol binding to the receptor induces conformational changes in the hormone binding domain (HBD) of the receptor. A 73 kDa protein (p73) remains bound to HBD under hormone free conditions. The hormone induced conformational changes in the HBD move the 73 kDa protein close to the p28. The protein-protein interaction between p73 and p28 leads to the dissociation of p28 from the NLS, thereby facilitating the binding of p55 to the NLS (Padma et al, 2000; Padma & Thampan, 2000).

Estrogen receptor activation factor (E-RAF) (Thampan & Clark, 1981; Thampan, 1987, 1989) has its primary cellular location at the endoplasmic reti-
Fig. 2—Potential sites for phytoestrogen action in an estrogen target cell. (1) Internalization of the plasma membrane localized non-activated estrogen receptor (naER). The internalized naER interacts with the 58 kDa NLS binding protein that mediates the nuclear entry of naER. (2) Interaction of ERα with NLSbp55 prior to its nuclear entry. Estrogen binding to ERα with NLSbp55 prior to its nuclear entry. Estrogen binding to ERα is essential for this interaction to take place. The ERα remains bound to the estrogen responsive elements in the form of a homodimer. (3) Retention of tp66 - E-RAF complex at the ap55 of endoplasmic reticulum. ap55 is an estrogen binding protein that 'anchors' tp66 – E-RAF complex. tp66 transports E-RAF to the nucleus following their release from the ap55. The naER and E-RAF form a heterodimer within the nucleus. In this form, it is being proposed that, the naER binds to the RNA polymerase II while the E-RAF binds to the DNA. Deglycosylation of naER leads to the transformation of the naER to nuclear estrogen receptor II which is a RNA binding protein. Possibly once the transcription is initiated the naER dissociates from the RNA polymerase and binds to the pre mRNA in the mRNP. Recent evidence reveals that prior to the exit of the mRNP the nER II is located within the mRNP, bound to small nuclear ribonucleoproteins (snRNPs). (4) Nucleocytoplasmic transport of mRNP, mediated by nuclear estrogen receptor II (nER II). Estrogen binding to nER II initiates this movement of mRNP from the nucleus to the cytoplasm.

culum (Govind et al, 2003 a & b). A 55 kDa integral protein of the endoplasmic reticulum membrane, that binds estradiol with high affinity, anchors E-RAF to the endoplasmic reticulum, mediated by a 66 kDa protein. The estrogen binding anchor protein of the endoplasmic reticulum is the anchor protein 55 (ap55) and the 66 kDa protein that transports E-RAF to the nucleus is the transport protein 66 (tp66) that also recognizes the NLS signals on the E-RAF. In the presence of estradiol (7 nM or above) the ap55 keeps tp66-E-RAF complex anchored to it while in the presence of estradiol (below 7 nM), the ap55 changes its conformation, leading to the release of the tp66-E-RAF complex. The tp66 transports E-RAF to the nucleus. In this instance tp66 has the properties of both importin α and importin β. At the nuclear periphery tp66 remains ‘dockeed’ at a 38 kDa nuclear pore complex protein, npcp 38 (Govind et al, 2003b).

E-RAF, after its nuclear entry, dimerizes with the naER. It appears that the complex binds to the gene at the transcription initiation site, where E-RAF binds to the DNA and naER binds to the RNA polymerase.
Deglycosylation of naER by the naER transforming factor (Jaya & Thampan, 2000) leads to the transformation of naER to nuclear estrogen receptor II (nER II) that is incapable of dimerization with the E-RAF. E-RAF, dissociated from the heterodimer, destablizes the DNA, creating sites for the RNA polymerase to bind. nER II continues its association with the RNA polymerase and in the process phosphorylates two RNA polymerase subunits. After completing its role in transcription the nER II, being a RNA binding protein, finds its binding site initially on the mRNA and subsequently on the small nuclear RNAs (snRNAs). Within the snRNP the nER II interacts specifically with three proteins, p32, p55 and p66. Estradiol binding to nER II initiates a protein-protein interaction among nER II, p32 and p35, resulting in the activation of the inherent Mg $^{2+}$ ATPase activity of the complex (Sebastian & Thampan, 2002 a & b). This enhancement in the ATPase activity appears to be the immediate reason behind the hormone mediated nucleocytoplasmic transport of the mRNP (Sebastian & Thampan, 2002b).

Conclusion
Considerable information has accumulated concerning the molecular mechanisms of action of estrogen in reproductive and nonreproductive tissues. Since both long term genomic and rapid nongenomic mechanisms are involved in estrogen action, it is probable that physiological effects of phytoestrogens may also be mediated through these pathways. Due to the presence of a ligand binding site with a volume double that of 17β estradiol molecular volume, it has been observed that both ER$\alpha$ and ER$\beta$ can interact with a wide variety of molecules that have some structural similarity with 17β estradiol. At present there is considerable amount of epidemiological data available supporting the possible health benefits of phytoestrogens along with some results to the contrary. However, for the better utilization of the possible health benefits of phytoestrogen a deeper understanding of the mechanisms of action of these molecules is necessary. An illustration identifying the molecular events that require the physical presence of the hormone during the course of estrogen action has been presented. It is possible to develop assays for phytoestrogens based on these results. It is being proposed that the effectiveness of the test phytoestrogens at these individual molecular events may be confirmed prior to their utilization in any commercial endeavour.

Acknowledgement
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References


Govind A P et al, 2003 b, Estradiol dependent anchoring of the goat uterine estrogen receptor activation factor (E-RAF) at the Endoplasmic reticulum by a 55 kDa anchor protein (ap55), J Cell Biochem (in press).


Sebastian T & Thampan R V, 2002b. Nuclear estrogen receptor II (nER II) is involved in the estrogen-dependent ribonucleoprotein transport in the goat uterus: Isolation and characterization of three snRNP proteins, which bind to nER II. J Cell Biochem, 84, 227-236.


