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Review

Phytoestrogens as modulators of steroid action in target cells

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Abstract

Although numerous reports exist on the potential beneficial role of nutritional phytoestrogens in human health, their molecular mechanism in target cells is still not completely understood. Phytoestrogens promote estrogen and antiestrogen effects by interacting with numerous molecules, carrier proteins, enzymes and membrane and nuclear receptors, directly or indirectly involved in the transfer of estrogen signals. The hypothesis that the ERβ subtype plays a key role in antiproliferative effect of phytoestrogens, especially in breast cancer, is examined here.This review focus on the effects of phytoestrogens in developmental processes such as those linked to reproductive function, tumorigenesis and angiogenesis.

Keywords: Reviews; Phytoestrogens; Steroids

Contents

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1. Introduction

We generally think of estrogens as endogenous gonadal or adrenal hormones in mammals, but they can also originate from exogenous dietary plant sources. These naturally occurring, plant-derived estrogens, defined broadly as phytoestrogens, include the flavonoids (kaempferol, and quercitin), the isoflavonoids (genistein, daidzein, formonetin and equol), the lignans (enterolactone, enterodiol, and nordihydroguaiaretic acid (NDGA)), the coumestanes (coumestrol), the mycotoxins (zearalenol) and the stilbens (resveratrol). Several of them are ingested as precursors and then converted by the microflora of the mammalian gut. They are all polyphenols, and many of them are structurally similar to the natural and synthetic estrogens (Fig. 1). The concentrations of phytoestrogens in biological fluids are much higher than those of the endogenous steroid estrogens found under physiological conditions. For example, after the dietary ingestion of phytoestrogens they can reach very high concentrations, particularly in the urine of vegetarians (1000 times higher than those of total urinary steroid estrogens) and in the plasma (i.e., 1000 times higher than estradiol in pregnant women or in cord bloods and 10 000 to 100 000 times higher than estradiol concentrations during the menstrual cycle) [1–14].

Phytoestrogens have rather weak estrogenic activities (10^{-2}- to 10^{-3}-fold) compared to the circulating estrogens (17β-estradiol or estrone) [8,9,14], but their very high concentrations could account for the many biological effects attributed to them. Indeed, numerous epidemiological, clinical and experimental studies, both in vitro and in vivo, suggest that phytoestrogens could notably modulate the impact of estrogen on the target cells. However, there are conflicting data about the hormonal action of phytoestrogens, which probably results from the multifaceted effects of these compounds on the transfer of information mediated by estrogen, affecting biological functions such as proliferation, differentiation and protein synthesis in different target cells. The complexity of the influence of phytoestrogens at the cellular and molecular level is further increased by the fact that their effects are also dependent on the dose, the class to which they belong, the presence or absence of endogenous estrogens, the status of the receptor and the type of target tissue or cell considered. In addition to their effects on the transfer of steroid messages, they may affect the metabolism and biological activity of estrogens and fatty acids [1–5,9,15–18], as well as producing other effects, such as influencing the enzymes involved in signal transduction pathways [19,20] and gene transcription [21]. The phytoestrogens therefore exhibit many other activities, such as anti-oxidant, anti-proliferative, and anti-angiogenic properties, all of which could contribute to the potential anticancer, anti-osteoporosis and cardiovascular protective effects of phytoestrogens [6,8,9,12,14,22].

Many reviews elsewhere have considered the effects of phytoestrogens on osteoporosis and on the cardiovascular systems [8,9,13] and we will not therefore discuss them here. This review focuses on the effects of phytoestrogens at the various steps in the transfer of the steroid message, and summarizes some of the potential actions of phytoestrogens on hormone-dependent processes, such as the development of the female reproductive tract, and in angiogenesis and tumorigenesis (uterine and breast cancer). Future investigations which may be of particular relevance to fetal and neonatal development will also be discussed.

2. The role of phytoestrogens in the transfer of steroid signals

Phytoestrogens can act at several different levels: the biosynthesis and metabolism of steroids and fatty

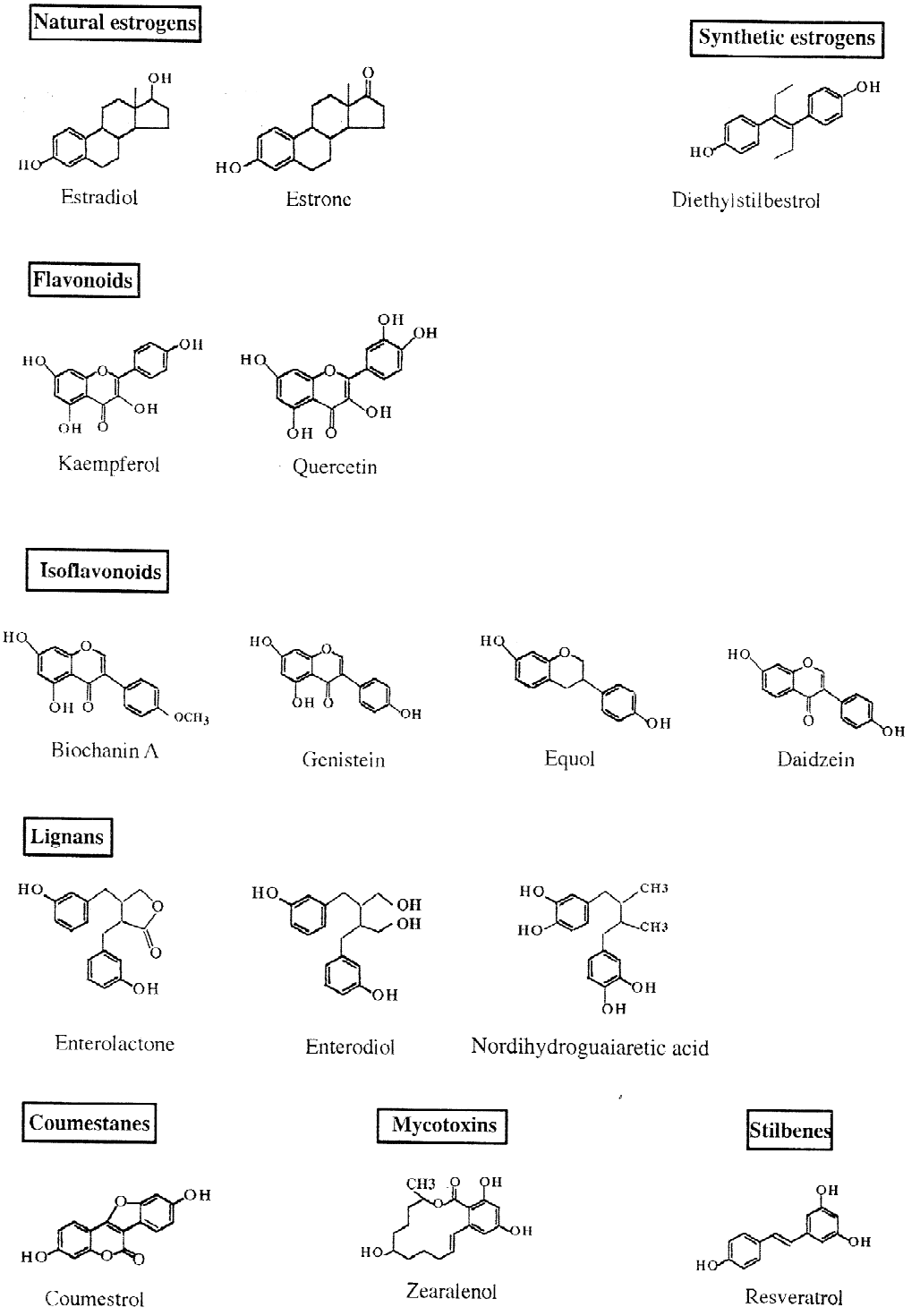

Fig. 1. Comparison of molecular structures of phytoestrogens and natural or synthetic estrogens.

acids, the serum steroid-binding proteins, the intracellular and transmembrane transfer of hormones to a membrane and nuclear receptors, the later modulating gene transcription.

2.1. Phytoestrogens and key enzymes involved in metabolism, signal transduction and gene transcription

While there is no evidence for any in vivo activity of aromatase-inhibiting flavonoids [23] several studies indicate that in vitro phytoestrogens inhibit human aromatase [7,8,14,24], 17β-hydroxysteroid dehydrogenase [7,8,24] and 5α-reductase [25,26]. They also affect the activities of cyclooxygenase [18] and lipooxygenase [16], and cholesterol 7α-hydroxylase, an enzyme implicated in the formation of primary biliary acid from cholesterol in the colon [14,27].

Modulating effects of phytoestrogens on the activities of enzymes of the signal transduction pathway have also been reported. Isoflavonoids inhibit the activities of tyrosine kinase [8,28], and protein kinase C [19,20,29]. Genistein inhibits both topoisomerase I and II activities [21,30], these enzymes catalyse topological changes in DNA and are required for DNA replication [8,14,31] and are also implicated in the gene transcription process.

2.2. Phytoestrogens and plasma binding proteins

Absorbed phytoestrogens are usually transported to their target cells by being bound to specific plasma carrier proteins, such as human sex steroid binding protein and the onco-fetal plasma protein, α-fetoprotein [32–36].

Sex steroid binding protein (SBP) has high, specific affinity for estrogens and androgens. About half of the circulating testosterone in men and 88% of the total estrogen in pregnant women are bound to SBP, the levels of which are greatly increased during pregnancy [37]. SBP has also been found in breast and prostate cancer cells [38,39]. Genistein has been shown to induce SBP synthesis by human hepatocytes in vitro [3,40]. Phytoestrogens have different, dose-dependent inhibitory effects on the binding of steroids to SBP [33,36]. Their relative effectiveness in displacing 17β-estradiol are: enterolactone≥ NDGA=equol>genistein>, enterodiol⋙daidzein; and for displacing testosterone: equol> enterolactone>NDGA>genistein⋙enterodiol and diadzein.

α-Fetoprotein (AFP) is a major serum glycoprotein synthesized during fetal life. This protein rapidly disappears after birth, and its reappearance during adult life reflects the proliferation of abnormal cells such as hepatic or germ cell tumours [41]. Rat and mouse AFPs alone possess the ability to bind estrogens with high affinity [42,43], but share with human AFP the property of highly binding long-chain polyunsaturated fatty acids, such as arachidonic acid (C20:4 w-6) or docosahexaenoic acid (C22:6 w-3), precursors of prostanoids and leukotrienes [44,45]. Competition experiments indicate that phytoestrogens have differential inhibitory effects on the binding properties of both rat and human AFP [34,35]. The estrone and 17β-estradiol displacement efficiency of NDGA is greater than equol> enterolactone>enterodiol for high affinity binding sites of rat AFP; NDGA has also been shown to inhibit [^{3}H]C20:4 binding to rat and human AFP in a dose-dependent manner [34].

Phytoestrogens therefore compete with estrogens for the high affinity binding sites on human SBP and rat AFP, and with the polyunsaturated fatty acids for binding to rat and human AFP. Phytoestrogens display inhibitory effects at concentrations in the range of 0.5–50 μ*M*, which are similar to those found in urine. Hence, differing conformational transition states of the SBP and AFP proteins are observed, depending on their phytoestrogen environment [33,34]. Phytoestrogens therefore appear to be nutritional modulators of the physicochemical properties of AFP and SBP.

These findings raise the question of the molecular and cellular significance of the binding of phytoestrogens to human AFP and SBP. These interactions may have special significance in physiological and pathological situations, especially when the concentrations of these carrier proteins are modified. This is why an overview of the role played by such phytoestrogen–plasma protein interactions in hormone-dependent developmental processes such as pregnancy and breast cancer will be discussed later.

2.3. Phytoestrogens and membrane receptors

A variety of short-term estrogen effects (lasting a matter of seconds to minutes) on their target organs has been reported, and it has been proposed that plasma membrane resident forms of steroids receptors may mediate such non-genomic actions [46–48]. Few studies have demonstrated any direct effect of phytoestrogens on the membrane estrogen receptor, nevertheless, the interactions between phytoestrogens and carrier protein described above may, for example, influence the recognition of SBP and AFP by their own membrane receptors. Indeed, the steroid–SBP complex may interact with these membrane receptors, especially those of human breast cancer [49], decidua [50], and syncytiotrophoblast [51]. These interactions are the first sequence of events leading to a specific cell response, such as activation of signal transduction pathways, and the production of a second messenger (i.e., cAMP) [49,52,53].

2.4. Phytoestrogens and nuclear estrogen receptors (ERs)

The genomic actions of estrogens involve the binding of the hormone to a nuclear receptor, which binds to DNA on specific palindromic sequences, known as estrogen responsive elements (ERE), and triggers RNA-dependent protein synthesis.

Structurally, estrogen receptors belong to the nuclear receptor superfamily. The aminoterminal A/B domain is involved in transactivation of gene expression. The C-domain contains a two-zinc finger structure, which plays an important role in receptor dimerization and specific receptor–DNA interaction. The carboxyl-terminal ligand-binding domain (or E/F domain) is crucial for the binding of receptor specific ligands, nuclear translocation, receptor dimerization, and modulation of target gene expression in association with corepressors and coactivators [54,55]. Transactivation functions, AF1 and AF2, have been identified within the A/B and F regions, respectively [56]. ER phosphorylation is essential for both dimerization and gene transcription. The sites of phosphorylation (eight sites in the A/B and C/D regions, and one in the ligand-binding domain E/F) are the targets for various kinases such as protein kinase C and tyrosine kinase, the activities of which, as seen above, may be modulated by phytoestrogens [14,55,57–59].

The recent demonstration of the existence of two receptor subtypes (ERα and ERβ) reveals that the biological action of estrogen and related phytoestrogens is more complex than previously thought. Human ERα and ERβ derive from two specific genes, and differ in both the C-terminal ligand-binding and the N terminal transactivation domains. The highest homology is in the DNA binding domains [60–62]. Both forms have been identified in women's breast, uterus, ovary and in the blood vessels, but the proportions of the α and β forms vary according to the estrogen target tissues and their physiological or pathological status. ERα is predominant in the testis, kidney, adrenal and non-pregnant myometrium, whereas ERβ is predominant in human brain, thymus, bladder, prostate, lung, bone and pregnant term myometrium. Ligand binding analysis indicates that the affinity of hERβ for 17β-estradiol is one-quarter that of hERα [63,64].

The estrogenic activity of phytoestrogens has been investigated in in vitro test systems: (i) competitive binding assay with ER from various tissues or the ERα/or ERβ recombinant human receptors, and (ii) transient gene expression assay using cotransfection experiments with hERα or hERβ cDNA in the presence of an estrogen-dependent luciferase reporter gene-containing plasmid [61,63]. Because each of these two estrogen receptors may influence the functions of the other, the effect of estrogen in tissues where they are coexpressed is very complex, and the resulting changes in physiological functions can be difficult to interpret when their proportions are modified.

2.4.1. Effects of phytoestrogens on ER binding properties

Several studies indicate that phytoestrogens (NDGA, genistein, coumestrol, equol, daidzein) inhibit the specific binding of 17β-estradiol to ER in rat and human uterus and in human mammary gland in a dose-dependent manner (0.1–10 μM) [32,65–67]. ERα and ERβ have distinct physical characteristics in the rodent ventral prostate, ERβ sedimenting as a broad 4S peak, whereas ERα sediments as an 8S peak in a sucrose-gradient [68]; the data also suggest that estrogen nuclear 4S type II binding sites (bio-

flavonoid receptors) [67] are selectively inhibited by phytoestrogens [69]. Recently, differential binding of phytoestrogens to recombinant ERα and ERβ has been investigated by Kuiper's group [61,63]. The relative binding affinities of phytoestrogens to the α and β estrogen receptor subtypes are lower than that of 17β-estradiol, as measured both in solid-phase and solubilized receptor ligand-binding systems. However, these compounds compete with 17β-estradiol for binding to either or both ER subtypes. Both coumestrol and genistein have significantly higher affinity for ERβ protein than for ERα (a 7-fold and 20–30-fold difference, respectively), whereas zearalenol and resveratrol only display a very slight difference in their affinity for the two receptor subtypes [63]. The position and number of the hydroxyl substituents on the flavone or isoflavone molecules seem to determine the ER binding affinity. For example, genistein has a particular high binding affinity for ERβ, but elimination of one OH group (daidzein, biochaninA) or two OH groups (formonetin) leads to a considerable loss of binding affinity [61].

The affinity of a ligand for a receptor and the conformational change induced by the ligand after being bound to the receptor are important parameters that determine both the transcriptional efficacy and agonist/antagonist activities of a ligand.

2.4.2. Effects of phytoestrogens on the transcriptional activity of ER subtypes

Despite their lower binding affinities for ER than 17β-estradiol, phytoestrogens exhibit estrogenic activities in transactivation assays with ERα and ERβ. Using human embryonic kidney cells and transient co-transfection with a luciferase reporter gene construct containing three copies of a consensus ERE in front of a TATA-box, together with hERα or hERβ expression plasmids, Kuiper et al. [61,63] have shown that phytoestrogens stimulate the transcriptional activity of both ER subtypes at concentrations of 1–10 n*M*. The ranking of their estrogenic potency depends upon the ER subtype: 17β estradiol ≫ zearalenol = coumestrol > genistein > daidzein > biochanin A>quercitin for ERα and 17β-estradiol ≫ genistein = coumestrol > zearalenol > daidzein>biochaninA>quercitin for ERβ [61]. At higher concentrations (100 n*M*), phytoestrogens are able to generate a response of the same magnitude as that induced by the physiological hormone, and at 1000 n*M* (a concentration reached in human serum after the consumption of food containing large amounts of soybean protein extracts) the estrogenic potency of genistein was greater than that of 17β-estradiol (E2).

The potency of genistein in hERβ expressing cells (human 293 kidney epithelial cell line) was 4–5-fold higher than that hERα expressing cells, and in contrast to expectations, genistein was only a partial agonist via the β receptor (60–70% of E2), but a full agonist when the transcriptional response was mediated by ERα (107–130% of E2) [55,70].

The gene transcription mediated by a receptor after the binding of a ligand depends on the conformational change in the receptor induced by the ligand and on the subsequent events, including receptor dimerization, receptor–DNA interaction, formation of a preinitiation complex, and recruitment of distinct subsets of coactivators/corepressors and other transcription factors which may depend on changes in the cellular environment [54,55]; this makes it possible to discriminate between estrogen agonist and antagonist actions. In the case of genistein, crystallographic studies with the ligand-binding domain of ERβ reveal that, helix 12 in the binding cavity does not adopt an agonist conformation with genistein, but occupies a position more similar to that seen in the presence of an antagonist. Thus, differences between the ligand-binding cavity of hERα and of hERβ could account for the receptor-selective character of genistein, in particular for its α- versus β-selective difference in efficacy. However, another explanation might be that ERβ has somewhat different requirements for coactivators than hERα, a difference that may only become apparent with particular ligands such as genistein. Thus, recent reports indicate that isoflavones elicit distinct transcriptional actions from estrogens by selectively recruiting coregulators to ERβ [71].

3. Biological effects of phytoestrogens

3.1. Reproductive effects

Attention has focused on the possible adverse effects of phytoestrogens on fertility or resulting from in utero and post-natal exposure.

3.1.1. Animal fertility

Observations made from 1940 to 1970 have reported that the ingestion of high levels of phytoestrogens by animals led to relatively consistent adverse effects on reproduction which were more marked in females than in males. The consumption of high levels of isoflavones, such as those found in clover, led to infertility in cattle, sheep and rodents (guinea-pig, rabbit and mice), as well as endometrial hyperplasia in sheep and guinea-pig [12]. The compound responsible for these effects was found to be equol, which is formed by the bacterial metabolism of formononectin in the digestive tract. Later, dietary phytoestrogens (especially daidzein and genistein) were also implicated in reproductive failure in captive cheetahs in North America [72] and in several bioassays, including uterotrophic activity. These effects involve the inhibition of the secretion of hypothalamic LHRH and pituitary luteinizing hormone (LH), modification of the ovulatory process, and inhibition of progesterone production, which could be responsible for the high abortion rate observed in ewes exposed to estrogenic pasture [73,74].

Recent studies also indicate that ex vivo genistein, daldzein and equol modulate rat uterine contractile activity. Various mechanisms of action could explain these effects. It seems possible that phytoestrogens could bind to ERs and induce anti-estrogenic effects or very weak estrogenic effects modifying the responsiveness of the uterus to contractile agents [75].

3.1.2. Human fertility (premenopausal)

Although dietary phytoestrogens have been implicated in adverse effects upon fertility in various animals, there are no published reports of such effects in human populations consuming large amounts of these substances. The reproductive capacity of Asian women who consumed large amounts of soy products does not appear to be affected [76]. However, Cassidy et al. established that isoflavone-rich diets can exert hormonal effects and interfere with the regulation of the menstrual cycle in premenopausal women (increased-length of the menstrual cycle and/or delayed menstruation in premenopausal women, and reduced levels of LH, follicle stimulating hormone (FSH) and progesterone) [77,78]. Recently, dietary phtyoestrogens have been shown to decrease serum 17β-estradiol [79], a finding which could also be related to changes in the length of the menstrual cycle. At this time, it would appear to be premature to specifically attribute the longer menstrual cycle of Asian women to their high intake of phytoestrogen, although breast cancer patients have been shown to have a significantly shorter menstrual cycle than control subjects [80].

3.1.3. Pregnancy and fetal development

In utero: possible adverse effects in male rodents exposed to estrogenic substances during early life includes impaired semen quality, increases in the incidence of congenital malformations (such as cryptorchidism) and of testicular cancer [81–83]. In female rodents exposed in utero to coumestrol or isoflavones at concentrations compatible with dietary content, the main adverse effects were those on the development of the reproductive tract (shorter anogenital distance), and on the maturation of neuroendocrine control of ovulation and puberty [73,81–86].

In neonatal rats, the administration of isoflavones led to hormonal changes, such as an increased or decreased LH surge depending on the dose (10 or 50–1000 μg/newborn animal, respectively [73,84]. Abnormal sexual behavior (delay in mating receptive females) was only observed in males exposed neonatally to coumestrol, and not in those exposed to other phytoestrogens. The effects of neonatal exposure to coumestrol and equol on the development of the female rat reproductive tract have also been examined [82]. Premature uterine gland development and increased uterine weight were observed with 100 μ*M* coumestrol on post-natal days 1–5; at later ages, the uterine weight was significant lower, and a severe reduction in ER levels was observed. Equol (100 μ*M*) reduced the uterine weight at later ages, but did not affect ER levels. When given on post-natal days 10–14, both phytoestrogens caused dose-dependent inhibition of uterine gland growth [8].

In humans, unfortunately, there is little information concerning the effects of in utero exposure to phytoestrogens. Effects such as those described above do not appear to have been observed in the offspring of populations habitually consuming a diet containing high levels of phytoestrogens, such as the Chinese and Japanese [12], although a trans-placental transmission of phytoestrogens is demonstrated [87,88]. However, it is appealing to think that

phytoestrogens binding to a plasma carrier protein such as AFP or SBP could contribute to their uptake by fetal tissues via transplacental exchanges with the mother. Consequently, particular attention should be given to the effect of these environmental factors in modulating the steroid hormone message during pregnancy. In particular, their presence and potential role at the maternal–fetal interface should be investigated, since recent studies have detected high concentrations of AFP and SBP in the maternal intervillous blood space from pregnant women at term [89]. The maternal intervillous blood is in direct contact with the fetal trophoblast and forms a vital interface between mother and fetus, so it will be of particular interest to look at the phytoestrogen status in this pivotal compartment. Moreover, the human myometrium which mainly expresses ERβ in late pregnancy [90] appears to be a target tissue for phytoestrogens like genistein that preferentially bind to this receptor subtype.

These findings suggest that local changes in the concentration of phytoestrogens at the materno–fetal interface in late gestation may alter estrogenic signals and thus play a crucial role in mediating immunological, metabolic and endocrine processes, contributing to a subtle control of some aspects of parturition. Of course, this hypothesis needs careful analysis, but it focusses attention on the considerable impact that phytoestrogens could have on the multifactorial processes involved in pregnancy and parturition. The post-natal development of young babies exposed to extremely high levels of phytoestrogens through soy-based infant formulas would also have to be considered.

3.1.4. Post-menopausal women

The effectiveness of foods containing phytoestrogens in treating symptoms of the menopause, especially in reducing hot flushes, is controversial [12,13,91–93]. There are variations in response within studies, because the study population, the soy products used and the design of the trials (particularly with respect to the duration of exposure) are not always consistent. Thus, a double-blind placebo controlled trial in 104 post-menopausal women demonstrated a significant reduction in the frequency of hot flushes in the group consuming soy (60 g/day for 12 weeks) [92], whereas the recent Mayo Clinic study [93] shows that the effect of soy on hot flushes was minimal compared to the reduction achieved by means of estrogen replacement therapy. These results could be linked to the strong placebo effect observed by other authors [13]. Recent preliminary data also suggest that phytoestrogens can be useful in preventing post-menopausal osteoporosis [94]. To date, the limited number of studies prevents a categorical conclusion and large-scale studies should be undertaken to assess the exposure of the various western populations, taking into account that soy is increasingly used in human food for its potential beneficial effects and that additional intake occurs during estrogen replacement therapy.

3.2. Uterine and breast cancer

3.2.1. Epidemiological and in vivo studies

Epidemiological studies indicate that dietary factors contribute to about one-third of potentially preventable cancers especially of the breast, endometrium, prostate, colon, rectum, stomach and lung [12,13,95]. Breast, prostate, and endometrial cancers all belong to a group of hormone-dependent cancers that, like colon cancer and coronary heart disease, have a lower incidence in Asia than in Western countries. Here we will only discuss hormone-dependent cancers in women (uterine, breast).

3.2.1.1. Uterine cancer. A high incidence of uterine adenocarcinoma after neonatal exposure to diethylstilbestrol (DES) has been described previously in humans and in animal models. In contrast, few studies have investigated the carcinogenic potential of phytoestrogens in animal models. A recent study has shown a similar increase of uterine adenocarcinoma in adult life in mice [96] treated neonatally (on days 1–5) with equivalent doses of genistein (50 mg/kg per day) or DES (0.00 1 mg/kg per day) (incidence 31 and 35%, respectively). These findings suggest that genistein could be carcinogenic if exposure occurs during critical periods of differentiation. Genistein could also increase uterine weight and up-regulate gene expression in tumor-bearing animals, as compared to ovariectomized animals [97]. Both estrogen receptor subtypes are present in normal and tumoral human endometrium ([98–100], Perrot-Applanat unpublished observations); their in-

volvement in phytoestrogen action remains to be established.

3.2.1.2. Breast cancer. There is evidence that steroid hormones are involved in the development and function of the mammary gland, as well as in controlling hormone-sensitive breast cancer growth. The crucial role of 17β-estradiol in the process of breast cancer was proven by the significant protective response observed in post-menopausal women who had been treated with the anti-estrogen, 4-hydroxytamoxifen, resulting in a decrease in mortality and incidence [101]. Numerous plants have been investigated in the search for cancer chemopreventive agents [102]. Thus, a number of epidemiological studies have suggested that the dietary intake of phytoestrogens decrease the risk of breast cancer in humans [76,103]. Indeed, the incidence of human breast cancers is lower in Asian countries, where foods contain high levels of phytoestrogens, than in Western countries. This is in favor of a dietary influence, but it is possible that this effect could be the result of a genetic adaptation process occurring over several generations. The study by Ingram et al. [104] also shows that increased urinary excretion of isoflavones and lignans is associated with a substantial reduction in breast cancer risk. Likewise, the consumption of foods rich in phytoestrogens, which are common in the Mexican diet, appears to play a protective role and reduces the incidence of breast cancer [105]. By contrast, phytoestrogens appears to have little effect on breast cancer risk in a multiethnic population [106].

Phytoestrogens exhibit biological properties that are quite distinct from the action of classic estrogens. However, their possible protective effect in human breast cancer is still subject to debate, and the mechanisms involved remain to be clarified [12,76]. Whether phytoestrogens should not be recommended for women with estrogen-positive tumors is still uncertain in the absence of scientific data. Due to genetic variations, some of the environmental estrogens could be carcinogenic in some individuals while protective in others [107].

Likewise, the effects of in utero exposure to phytoestrogens are controversial, and there is no information concerning the incidence of soya-based infant formula milks on the risk of breast cancer. Some authors report that in utero exposure of rodents to phytoestrogens has anti-proliferative effects on mammary tumor formation [13], while others report that in utero exposure to genistein at physiological levels increased proliferation in mammary tumors subsequently induced by the carcinogen dimethylbenz[*a*]anthracene in young rats at birth [108].

In neonatal rats, the administration of genistein at a level reported to be within the range of human dietary intake led to a reduction in mammary tumor formation in adult life when tumors were induced by the carcinogen [109].

In vivo studies using human tumor xenograft tissue indicate that phytoestrogens stimulate growth of estrogen-dependent human breast cancer cells in the athymic mouse tumor implant model [110].

3.2.2. In vitro studies

In vitro, phytoestrogens can act as estrogen agonists or antagonists on cancer breast cells, depending on numerous factors such as the nature and dose of the compound, the status of the receptor, the presence of endogenous estrogen, growth factors and cytokines, and on various negative and positive transcription cofactors [9,111–113]. It has been observed that estrogen-dependent human breast cancer cells (MCF7 cell line) can adapt to low levels of estrogens by enhancing their sensitivity to 17β-estradiol [114].

Phytoestrogens have been shown to have a variety of effects on human breast cancer cell growth. At low concentrations, genistein stimulates proliferation via receptor-mediated pathways in ER-positive breast cancer cells (estrogen-dependent MCF7 cells). In the same way, Welshons et al. [115] have shown that enterolactone, enterodiol and equol are able to stimulate MCF7 cell growth, and that their effect is antagonized by tamoxifen. However, at high concentrations (>10 μM) or in the presence of 17β-estradiol, phytoestrogens (genistein, enterolactone, NDGA, equol) significantly inhibit MCF7 cell growth [63,65,116–120]. Such anti-estrogenic effect of phytoestrogens was usually explained by competition with endogenous estrogens for receptor sites, which prevented estrogen-stimulated growth in mammals. Moreover, Wang et al. [118] have shown that prolonged exposure to genistein results in a decrease

in the ER mRNA level, leading to reduced responsiveness to endogenous estrogens.

The fact that phytoestrogens (genistein, daidzein and biochamin A) inhibited the growth of ER-negative breast derived cells (MDA-MB-231 cells) [116,121] at low and high concentrations suggests that other receptor-independent mechanisms may be involved, such as an increase in the metabolic clearance of estrogen, the stimulation of SBP synthesis, the inhibition of aromatase, modulation of the production of prostaglandins and leukotrienes involved in carcinogenesis [122], or the inhibition of protein tyrosine kinases and DNA topoisomerases. It was also observed in breast cancer lines, that the inhibitory effect of genistein on cell proliferation was associated with the arrest of the cell cycle in the G2/M phase, followed by apoptosis [123–126].

Since many recurrent breast tumors are both ER negative and multidrug resistant, the ability of genistein to inhibit the growth of breast cancer cells independently of ER status and multidrug resistance, makes it an attractive candidate for clinical use [116]. A synergistic action has been observed between genistein and w-3 fatty acids, which are known to have anti-angiogenic properties and are also a major component of the Asian diet [127,128]. The effectiveness of genistein compared to other classes of anticancer compounds including antiestrogens, retinoids, monoterpenes, and tyrosine kinase inhibitors remains to be evaluated [129].

3.2.3. ERα/ERβ status in breast tumors and human breast cell lines

Using immunocytochemical techniques, the expression of ERα and ERβ subtypes has been reported in epithelial cells of alveoli and ducts as well as in stroma cells of the normal human mammary gland [130]. ERα and ERβ mRNA subtypes were detected in breast tumor cell lines and in breast tumors. The level of their expression appears to vary widely among tumor samples and between cell lines, and the expression of ERβ was not correlated with that of ERα [131,132]. Although the low level of expression of the alternatively spliced ER transcripts in human breast cancer suggests that they may not be a determinant in receptor function [133], quantitative analysis indicates changes in the proportion of ERβ mRNA variants during the breast carcinogenesis [134].

Interestingly, both ERα-positive and -negative cell lines express ERβ mRNA, whereas only some breast tumors and some cell lines coexpress ERβ and ERα mRNA [131] (Lozano and Perrot-Applanat submitted results). In ER-positive primary breast tumors, a higher ERα/ERβ ratio was observed than in normal adjacent tissue. Higher ERα mRNA expression in the tumors than in the normal tissue and lower ERβ mRNA expression in some tumors only, suggest that altered ERα and ERβ gene expression plays a role during the development of breast tumors [135]. The decreased expression of ERβ observed between normal and neoplastic tissues could be one of the events leading to uncontrolled cell proliferation [134,136]. These findings are compatible with the hypothesis that ERα is mainly involved in promoting cell proliferation, whereas ERβ has a protective effect on this process [137]. Indeed, recent data provide evidence that ERβ inhibits the proliferation and invasion of breast cancer cells [138]. Considering that phytoestrogens, particularly genistein, bind to ERβ with five to 20 times more affinity than to ERα, it is likely that part of the antiestrogenic effect of these compounds in suppressing breast tumor growth occurs via the ERβ pathway [63,139]. While, in MCF-7 breast cancer cells, genistein binds to ERβ with almost the same efficiency as 17β-estradiol, the fact that much higher concentrations of isoflavones and derivatives are required to induce transcription and stimulate cell growth than for binding to ER subtypes may explain why these compounds help to reduce the risk of cancer [64].

Phytoalexins such as glyceollins (isoflavonoid derivatives) have been reported to have a marked anti-estrogenic effect on the ERα signalling pathway, which correlates with a comparable suppression of 17β-estradiol-induced proliferation in MCF-7 cells. In the light of the observed estrogenic effect of coumestrol, and daidzein, the contrasting lack of agonistic activity of the glyceollins is of particular interest [140]. Resveratrol, a phytoalexin with anti-inflammatory activity, mainly found in grapes, inhibits the development of preneoplastic lesions in a carcinogen-treated mouse mammary gland model [141]. Cell proliferation of MCF-7 and uterine

Ishikawa cells or recipient cells transfected with ERα or ERβ, but not in T47D [142], is reduced by high concentrations of resveratrol (≥100 μ*M*) [142–145]. The preferential binding of isoflavones to ERβ rather than ERα, in contrast to the similar binding affinities of resveratrol for both receptors may partly account for these differing results. Resveratrol also inhibits the activation of protein kinase C, preceding the activation of cyclooxygenase-2 and gene transcription [29].

3.3. Angiogenesis and endothelial cell proliferation

Anti-angiogenic effects have also been proposed as a possible mechanism for the anti-tumor activity of soy isoflavones [146,147].

Angiogenesis (i.e., the generation of new capillaries) is usually restricted to a few circumstances in the adult healthy organism, including wound healing and the formation of the corpus luteum, endometrium and placenta. These conditions represent ordered, tightly regulated, and self-limiting processes, and are the result of the well-balanced activity of inhibitors and activators of angiogenesis. The most clinically important manifestation of pathological angiogenesis is that induced by solid tumors [148]. Well-vascularized tumors expand both locally and by metastasis, whereas avascular tumors do not grow beyond a diameter of 1–2 mm [149], probably due to the low diffusion of nutrients.

Angiogenesis critically depends on several steps, including the degradation of the endothelial cell basal membrane, the migration and proliferation of endothelial cells and their organization into capillary tubes. These processes depend on the secretion of angiogenic stimuli, such as vascular endothelial growth factor (VEGF) and of matrix metalloproteases (MMP) which degrade the extracellular matrix [148]. VEGF is a polypeptide secreted by a large number of cells, which is among the most potent and specific known angiogenic factors in vivo and which increases microvascular permeability [148,150]. VEGF is also a chemotactic factor for monocytes [151].

Factors which up-regulate VEGF in various cells include hypoxia, multiple growth factors and cytokines [148], recently, our group [152,153] and other groups [154–156] have reported that VEGF expression is stimulated both in vivo and in vitro by 17β-estradiol in the uterus, in MCF7 and in vascular smooth muscle cells [153]. These findings indicate that the hormone may modulate angiogenesis by increasing VEGF expression. We still do not know whether the induction of VEGF is mediated by ERα and ERβ in normal tissues, although ERα is known to stimulate VEGF transcription in uterine cancer cells [157]. However, studies of estrogen receptor knock-out mice lacking ERα have revealed that 17β-estradiol induces angiogenesis, a process requiring estrogen receptors [158], presumably ERβ. In this case, 17β-estradiol also exerts a direct proliferative effect on human endothelial cells in vitro [159].

The first evidence of the anti-angiogenic activity of phytoestrogens was provided in vitro by Fotsis et al. [146], who screened the activity of compounds extracted from the urine of human subjects consuming a plant-based diet. This work led to the identification of the isoflavonoid genistein, as a potent inhibitor of endothelial cell proliferation and in vitro angiogenesis, producing half maximal inhibition at concentrations of 5 and 10 m*M*, respectively [146,147]. Genistein also markedly reduces the degradation of the extracellular matrix, by decreasing both FGFb-stimulated and basal levels of urokinase plasminogen activator and plasminogen activator inhibitor activity in endothelial cells [147]. The same authors extended these observations by investigating the antiangiogenic effects of flavonoids and shown that they inhibit the FGF-induced proliferation of endothelial cells at half-maximal concentrations in the low micromolar range [147]. The inhibitory activity of three of these flavonoids(3-hydroxyflavone, 3′,4′-dihydroxyflavone and luteolin) was approximately 2–3-fold stronger than that of isomeric genistein. These substances also inhibit in vitro angiogenesis on three-dimensional collagen, and some of them reduce invasion to a greater extent than the same concentration of genistein [147]. Recently, silymarin, another flavonoid, used clinically as a liver detoxicant has also been reported to inhibit several angiogenic responses, including endothelial cell growth, MMP-2 expression and in vitro angiogenesis [160].

Some in vivo studies [161–165], although not all [166–169], have reported that pure isoflavones inhibit the tumorigenesis of transplantable tumors in

animal models. Several possible mechanisms have been proposed for the anti-tumor activity of these compounds, including antiangiogenesis properties. The presence of significant concentrations of soy isoflavones in the urine has provided the rationale for investigating their effects on angiogenesis in bladder cancer. Analysis of vascular density has shown that genistein, daidzein and biochanin A inhibit the growth and reduce the angiogenesis of transplantable bladder cancer in mice [161].

Thus, in vitro and in vivo studies have both suggested that isoflavonoids and flavonoids exert multiple suppressive effects on angiogenesis, including the inhibition of several kinases (tyrosine kinase, protein kinase C, 1-phosphatidylinositol kinase, cdc2 and cyclin-dependent kinases) and the down-regulation of MMP-2. However, the mechanisms whereby soy products alter the angiogenic switch and the balance between angiogenic growth factors and inhibitors in vivo are still under investigation. Experiments with transplantable cancers placed orthopically within the same normal tissue may provide the preclinical data required to extend these observations to human trials.

3.4. Anti-oxidant efficacy of phytoestrogens

Anti-oxidant activity is a fundamental property underlying a wide range of biological effects, including: anti-bacterial, anti-viral, anti-inflammatory, anti-allergic, anti-thrombotic, antimutagenicity, anti-carcinogenicity, anti-aging, and vasodilatory effects [20,169,170].

The polyphenolic nature of phytoestrogens gives them the ability to act as anti-oxidants, and thus to inhibit or delay the oxidation of other molecules by preventing the initiation or propagation of oxidizing chain reactions. The anti-oxidant activity of phytoestrogens has been reported both in vitro and in vivo [22], and may help to lower the risk of cancer by protecting cells, for example, against lipid peroxidation and modulating the production of the prostaglandins and leukotrienes involved in carcinogenesis [122]. Other authors [171,172] reported that isoflavones inhibit lipoxygenase action and prevent peroxidative hemolysis of sheep erythrocytes in vitro. Wei et al. [173] found that genistein is a potent inhibitor of hydrogen peroxide production in HL60 cells and an inhibitor of the generation of superoxide anion by xanthine/xanthine oxidase. Genistein has also been shown in vivo to increase the activities of antioxidant enzymes in mice: catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase [22]. The human placenta may be a source of lipid peroxides [174], and a shift in the oxidant/antioxidant balance in the placenta and plasma may lead to diseases such as preeclampsia [175,176]. It has been suggested that such disorders may be due to placental–maternal antioxidant deficiencies. Does this mean that the anti-oxidant properties of phytoestrogens could have a potentially beneficial effect in this case?

4. Conclusion

Purely on the basis of the effects of phytoestrogens summarized in this review, it is tempting to speculate that the interaction of phytoestrogens with carrier plasma proteins (SBP, AFP) and estrogen receptors (ERα/ERβ) may have endocrinological, pharmacological and nutritional significance in physiological and pathological situations, in particular when the concentrations of these proteins are modified.The most obvious areas where phytoestrogens may be important appears to be in modulating the actions of the endogenous estrogens, the polyunsaturated fatty acids which are precursors of eicosanoids, and VEGF, since all these signalling factors are actively involved in the mechanisms governing vital processes, such as pregnancy, angiogenesis and tumorigenesis.

Phytoestrogens have a wide range of hormonal and non-hormonal activities that could provide plausible mechanisms for the potential health benefits of a high-phytoestrogen diet and this has led to the exponential increase of interest in the possible clinical importance of phytoestrogens. However, clinical evidence to support many of the currently claimed health benefits of phytoestrogens remains to be established and although promising, current data are not sufficient to support dietary recommendations for individual phytoestrogens. Over their potential to prevent and/or treat many hormone-dependent diseases, it is difficult to predict the in vivo effects of phytoestrogens, because the route of administration, the chemical form of the phytoestrogen, its metabolism, bioavailability, the half-life, the timing and

level of exposure, the intrinsic estrogenic status and the non-hormonal secondary mediated actions of phytoestrogens, all have to be taken into account when designing clinical studies to investigate their actions. It is likely that the beneficial effects of these compounds in vivo, are the combined result of multiple genomic and non-genomic effects. Indeed, further research is needed to evaluate the effect of phytoestrogens at the physiological and pharmacological levels, and to determine their effective doses for beneficial or harmful effects, as well as to evaluate their interactions with other dietary compounds, such as for example polyunsaturated fatty acids (w-6/w-3).

In conclusion, looking at Monet's famous painting ''*La femme à l'ombrelle*'' (*The woman with the parasol*) in the Louvre Museum in Paris, let us hope, just as the beautiful woman is shielded by her parasol and the plants surrounding her, that the ''Phytoestrogen ecology of steroid plasma carrier protein and receptors'' may provide her with another, more long-lasting, form of protection.

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