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THE USE OF ESTROGEN RECEPTORS FOR EVALUATION OF PHYTOESTROGENS ACTIVITIES IN MAMMALS

Marek Snochowski, Katarzyna Romanowicz*

The Kielanowski Institute of Animal Physiology and Nutrition of the Polish Academy of Sciences, Jabłonna, Poland

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Phytoestrogens represent a distinguished group of phytochemicals whose biological effect in mammals is clinically similar to that of exogenous estrogens. These substances may directly interfere with estrogen receptors (ER) regulated processes essential for normal physiological functions of mammalian organism. Therefore, in the presented paper we have summarized current information on the occurrence of phytoestrogens and available methods for estrogenic activity determination. We have also included data on tissue distribution and molecular characteristics of different ER subtypes, as well as, data on their interactions with phytoestrogens. Considering the important role of intracrine estrogen regulatory systems have described in separated part a ligand binding receptor method suitable for tissue level analysis. The example of experimental protocol used for determination of dietary phytoestrogens binding affinities to ER from the pituitary, uterus and thyroid gland collected from anoestrous ewes is included. The presented data fully confirm that dominant effects of phytoestrogens in mammals are caused by their direct interaction with ERs. The existence of functionally different multiple forms of ERs do not allow to create a single detection system for evaluation of phytoestrogens activity. The measurements performed using defined ER isoform result in information on its intracellular ligand activated mechanism of ER action. The analysis based on tissue ER extracts allows estimation of biologically effective concentrations of phytoestrogens. It is expected that the ongoing research on identification of ER inducible genes in vertebrate genomes may result in a more complex evaluation of estrogenic responses using proteins whose function is directly affected by phytoestrogens.

Abbreviations: AFs - transcription activation functions (AF-1, AF-2); DBD - DNA-binding domain; DPN - diarylpropionitrile selective ERβ agonist; ER - estrogen receptor; ERα - estrogen receptor alpha, systemic name: NR3A1; ERα46, Σ3, Σ4, Σ3-4 - ERα isoforms; ERβ - estrogen receptor beta, ERβ1, systemic name: NR3A2; ERβ2, ERβ1-δ3, ERβ2-δ3 - ERβ isoformes; ERE - estrogen receptor responsive DNA element; ERIN - estrogen receptor action indicator; HPLC - high performance liquid chromatography; hsp90, hsp70 - heat shock proteins; Kd - the apparent dissociation constant; Ki - inhibition constant; LBD - ligand binding domain; MS - mass spectrometry; Oestradiol - 17β-oestradiol, E_2; PPT - propyl pyrazole triol, selective ERα agonist; RBA - relative binding affinity; SERMs - selective estrogen receptor modulators; SRC - steroid receptor co-activator.

PHYTOESTROGENS

Phytoestrogens in plants. Phytoestrogens belong to two subclasses of polyphenols, the isoflavones and lignans out of over 100 000 low-molecular-mass natural plant products, known as secondary metabolites [Dixon & Sumner, 2003]. Flavones are antimicrobial natural plant products synthesized by shikimic acid linked pathway. Genistein, the most clinically relevant phytoestrogen, precursor of phytoalexins and phytoanticipins, is synthesized from the flavanone naringenin by a ring migration reaction catalysed by the n1Cytochrome P450 enzyme isoflavone synthase [Dixon & Ferreira, 2002]. The genes encoding those enzymes are recognized and have been experimentally used to produce transgenic plants as a source of biologically active isoflavones [Liu *et al.*, 2002]. The natural content of isoflavones highly differs among plant species, however, the amounts of phytoestrogens consumed by humans are evaluated according to their content in food and food products. Different databases have been created in the form suitable for the evaluation of phytoestrogen consumption. The most relevant are constructed with respect to locally available food resources, *e.g.* VENUS database [Kiely *et al.*, 2003], FINELI database [Valsta *et al.*, 2003], Nutrient Databank System [Dwyer *et al.*, 2003].

Screening of estrogenic activities. Phytoestrogens are included in the group of about 70 000 environmental chemicals tested for endocrine-disrupting activity. Different structure-activity relationship models for screening of chemicals have been developed, and the most recent MultiCASE expert system has been used to screen 2 526 compounds for their estrogen receptor (ER) binding activity [Klopman & Chakravarti, 2003]. However, it was emphasized that the program for screening which includes a screening battery involving a combination of at least eight *in vitro* and *in vivo* assays spanning a number of taxa, should be adjusted to current knowledge of mechanism of estrogen action [Daston *et al.*, 2003]. According to WHO/UNEP/ILO International Programme on Chemical Safety, high research priority has been given to the study of general mechanism of endocrine system [Damstra *et al.*, 2002] with

*Author's address for correspondence: Marek Snochowski, The Kielanowski Institute of Animal Physiology and Nutrition of the Polish Academy of Sciences, ul. Instytucka 3, 05-110 Jabłonna, Poland; tel.: (48 22) 7824422; fax: (48 22) 774 2038; e-mail: m.snochowski@ifzz.pan.pl

special emphasis for the recognition of the biological processes and tissue responses that changes according to the developmental stage of the organism [Damstra, 2003].

ESTROGEN RECEPTORS

The ER is considered to be evolutionary the oldest steroid receptor formed in basal vertebrates from the sequences of ancestral protein whose structure has been reconstructed. Two different genes encoding ER isoforms, ERα and ERβ, were found already in jawed vertebrates [Thornton, 2001]. According to McLachlan [2001], the ability of ER to bind different environmental substances seems to be the resistance of ancient signalling required, *e.g.* for symbiosis between plants and bacteria (cross-talk). Therefore, genistein exerting estrogen-like function can be described as a compound belonging to evolutionary ancient environmental signal contributing to human reproductive and developmental health [McLachlan, 2001]. In mammals, the predominant biological effects of estrogen are mediated through two types of intracellular receptors, ERα and ERβ, signed according to unified nomenclature system as NR3A1 and NR3A2 [Nuclear Receptors Nomenclature Committee, 1999]. Both ERs function as signal transducers, as well as, transcription factors to modulate expression of target genes. ERα and ERβ are distinct proteins encoded by separate genes located on different chromosomes, in human ERα is localized on chromosome 6, and ERβ on chromosome 14 [Enmark *et al.*, 1997].

Structure characteristics. The human ERα contains 595 amino acids while the most common form of ERβ 530 amino acids. They share a conserved structural and functional organization with other members of the nuclear receptor superfamily, including domains responsible for ligand binding, dimerization, DNA binding and transcriptional activation [Cheung *et al.*, 2003]. The DNA--binding domains (DBDs) of ERα and ERβ are highly homologous (96%), allowing both receptors to bind to the same estrogen response elements (EREs) and regulate similar sets of genes. The ligand-binding domains (LBDs) are also conserved (58% homology) with similar affinities of the two ERs for 17β-estradiol. In spite of these similarities, ERα and ERβ exhibit different affinities and responses with subsets of natural EREs and pharmacological ligands. Both ERs contain transcription activation functions (AFs), which allow the receptors to stimulate the transcription of estrogen-regulated genes. ERα contains two potent AFs, an N-terminal, ligand-independent activation function (AF-1) and a C-terminal, ligand-dependent activation function (AF-2). Both AFs in ERα are required for synergistic transcriptional activation, but can also function independently with certain cell type and promoter specificities. ERβ also contains an AF-2, but appears to have a weaker AF-1, which may possess repressive activity. The AF-2 domain of each receptor is regulated by ligand-induced changes in receptor conformation, but the activities of poorly conserved AF-1 domains are ligand-independent and can be modulated by phosphorylation. Transcriptional activities of ERs are dependent on a variety of co-regulatory proteins (co-activators, co-repressors) that are recruited by the receptors to estrogen-regulated promoters embedded in chromatin through direct or indirect interactions. These factors interact directly with the LBD in a ligand- and AF-2 dependent manner, including the steroid receptor co-activator (SRC) family of proteins and the Mediator-like complexes (TRAP, DRIP, ARC). Recently, Martin *et al.* [2003] described specific binding site within LBD of ERα for divalent metals and metal anions. The equilibrium Kd estimated for with cobalt and nickel was below 10 nmol/L and their binding blocked estradiol binding to receptor.

Intracellular action of ER. In the absence of hormone, ERs exist in a complex with chaperone proteins capable of high-affinity binding to steroid hormones. In response to ligand binding, ER undergoes conformational changes (activation), accompanied by dissociation of hsp90, hsp70 and other proteins, forming a ligand-occupied ER dimmer [Pratt & Toft, 1997]. ER may mediate the stimulation of target gene expression *via* different mechanisms. The most direct effect is realized by binding of ER diamer to a specific sequence called an estrogen response element (ERE) and interaction with coactivator proteins and components of the RNA polymerase II transcription initiation complex resulting in enhanced transcription. ER may also interact not directly with ERE but with another DNA-bound transcription factor in a way that stabilizes the DNA binding of that transcription factor and/or recruits coactivators to the complex stimulating gene expression [Klinge, 2000]. The cAMP-dependent activation of gene expression by ER has been described as ligand independent path which requires the presence of ERE for ERβ but not for ERα action [Coleman *et al.*, 2003]. The other mechanism described for response to 17β--estradiol is mediated by the ER signalling cascades at the cellular membrane and in the cytoplasm *via* various second messengers, such as receptor-mediated protein kinases. Nonnuclear ER signalling path has been described in relation to vasodilation, inhibition of response to vessel injury, limiting myocardial injury after infarction, and attenuating cardiac hypertrophy [Ho & Liao, 2002; Lösel *et al.*, 2003]. Both ERα and ERβ are coexpressed in a number of cells and form functional heterodimers. The biological roles of ERα/ERβ heterodimers in the presence of each respective homodimer are unknown. When coexpressed, ERβ exhibits an inhibitory action on ERα-mediated gene expression and in many instances opposes the actions of ERα. A number of ERα and ERβ isoforms have been also described as capable of altering the estrogen-mediated gene expression [Lindberg *et al.*, 2003; Matthews & Gustafsson, 2003].

ER isoforms. Both ERs genes can be transcribed from multiple promoters that give rise to mRNA variants that are expressed in cell and tissue specific manner. The presence and levels of specific ER isoform variants, along with receptor coactivator, corepressor and integrator proteins directly modulate overall nuclear ER activity [Shupnik, 2002]. Although the list of polymorphic variants is probably not yet complete, many of them have an already recognized physiological function.

ERα (NR3A1). The three isoforms of ERα (Σ3, 61.8 kDa; Σ4, 53 kDa; Σ3-4, 45 kDa) expressed as splice variants of full-

length ERα (68 kDa) are expected to play an important role during embryonic pituitary development [Pasqualini *et al.*, 2001]. The other ERα46, an amino-terminal truncated product of full-length ERα (ERα66) localized in the plasma membrane, cytosol, and nucleus of human endothelial cells has been recently described to play an important role in vascular-specific targeting of ER agonists [Li *et al.*, 2003]. Furthermore, the presence of multiple forms of ERα was detected in smooth muscle and endothelial cells of female rat cerebral blood vessels whose expression decreased after ovariectomy but significantly increased after chronic estrogen treatment [Stirone *et al.*, 2003].

ERβ (NR3A2). In addition to initially identified ERβ (ERβ1), three structural variants, ERβ2, ERß1-δ3 and ERβ2-δ3 were found in different rat tissues. The ERβ and ERβ2 are quantitatively dominant being expressed at equal levels in ovary, prostate, testis, pituitary, and muscle. They differ in ligand affinity (see below) and upon ligand binding can form heterodimers with ERα and ERβ [Petersen *et al.*, 1998; Saunders *et al.*, 2002].

Tissue distribution of ERs. The general distribution of ERs among different mammalian tissues seems to be similar among different species, although some differences between rodents and higher mammals have been described [Xu *et al.*, 2003]. The ERα is expressed primarily in the pituitary, uterus, liver, kidney and heart. The ERβ is expressed primarily in the ovary, prostate, bladder, lung, gastrointestinal tract and skin. A similar expression of both receptors occurs in the mammary glands, epididymis, thyroid, adrenals, bone and certain regions of the brain [*e.g.* Pelletier, 2000]. However, the cells expressing either ER type (or both) are not equally distributed within the tissue. So far, the most detailed localization of ERs has been described for the pituitary gland. In the rat, the expression of ERα was found in 45% of the lactotrophs and melanotrophs, 35% of the corticotrophs and folliculo-stellate cells, and 25% of the gonadotrophs. The distribution of ERβ expressing cells was similar but at lower level than ERα, while less than 10% of cells co-express ERα and ERβ [Mitchner *et al.*, 1998]. The ERs expression pattern is changing upon different physiological conditions and was found as differentially regulated [Vaillant *et al.*, 2002]. This includes the rapid nongenomic effect of 17β-estradiol on lactotrophs resulting in prolactin release demonstrated for normal rat pituitary [Christian & Morris, 2002].

In the brain both, ERs are distinctly distributed among specific neuronal structures [McEwen & Alves, 1999; Wang *et al.,* 2002]. ERα was found with the highest expression in the hippocampus while ERβ was detected in most areas of the brain [Taylor & Al-Azzawi, 2000]. The susceptibility of the brain to phytoestrogens is being successively recognized [Lephart *et al.*, 2002; Lyou *et al.*, 2002]. The selective agonistic action of genistein on ERβ in the hypothalamus has been demonstrated on rats [Patisaul *et al.*, 2002]. Recently, Hardy *et al.* [2003] used genistein experimentally to distinguish the ERα mediated action of 17β-estradiol in the retrochiasmatic area of the hypothalamus in ewe.

In the human ovary, ERα was localized in the granulosa cells, but not in the theca nor in the copora lutea, whereas ERβ was present in multiple cell types including granulosa cells in small, medium and large follicles, theca and corpora lutea [Taylor & Al-Azzawi, 2000]. Jefferson *et al.* [2002] reported an important observation that neonatal exposure to genistein induces at time of maturation enhanced ERα expression and multioocyte follicles in mouse ovary.

In the uterus, the cell specific expression of ERα and/or ERβ have been linked to functionally different activities involving different intracellular mechanisms [Frasor *et al.*, 2003; Kurita *et al.*, 2001; Monje *et al.*, 2001]. Moreover, ERβ and ERβ2 were found as differentially expressed with the intensity varying within the estrus cycle and my function as inhibitors of ERα transcriptional activity [Pillai *et al.*, 2002].

In the human testi, the ERβ forms seem to be major estrogenic regulators in contrast to rodents where ERα mRNA and protein were easily detected in both foetal and adult Leydig cells. In human foetal testicular tissue ERα is not expressed while in adult only in cell nuclei lining the efferent ductules. The ERβ and ERβ2 were positively detected during second trimester, as well as, in adult testis. The highest expression of ERβ was found in pachytene spermatocytes and round spermatids, while the highest density of ERβ2 protein was localized in Sertoli cells and spermatogonia. It is assumed that ERβ2 may have an inhibitory effect on ER action, protecting from adverse effects of estrogens [Gaskell *et al.*, 2003; Saunders *et al.*, 2002].

In the prostate, the ERα and ERβ have been localized within the dorsolateral part and were shown to be down-regulated by dietary genistein [Fritz *et al.*, 2002].

In the thyroid, both ERα and ERβ are expressed [Pelletier, 2000; Furlanetto *et al.*, 1999], but data on this tissue are rather limited. The immunohistochemical localization revealed that the presence of ER is mostly restricted to thyroid follicular/papillary type cells [Hiasa *et al.*, 1991], and according to Banu *et al.* [2002] they may play an important role during the time of maturation.

There are many other tissues where cell type ERs distribution have been not yet studied or the data were collected at a time when sufficiently specific methods were not available.

Ligand binding effects. The major endogenous estrogen (17β-oestradiol) binds equally ERα and ERβ, however, the structural differences within ligand binding domain are sufficient to recognize a number of different exogenous ligands. They may act as agonists or antagonists dependent on changes caused in the receptor protein conformation that may affect binding of coregulatory factors and/or its interaction with chromatin [Routledge *et al.*, 2000; Pearce *et al.*, 2003]. Although many of them are classified as selective estrogen receptor modulators (SERMs) the pure agonistic effect without interference with other ER isotype have been found for propyl pyrazole triol (PPT), ERα agonist, and diarylpropionitrile (DPN), ERβ agonist [Frasor *et al.*, 2003].

Among phytoestrogens present in soy-based diets the coumestrol and genistein are the most potent ER ligands with much higher affinity to ERβ then ERα. Their binding potency have been confirmed using pure ERs preparations obtained from transfected cells specifically expressing ERα, ERβ or ERβ2 [Kuiper *et al.*, 1997, 1998; Petersen *et al.*, 1998]. The obtained affinity values are summarized in Table 1 and clearly indicate that apart from ERα and ERβ also ERβ2 should be considered in evaluation of phytoestrogens [Petersen *et al.*, 1998].

TABLE 1. Phytoestrogens affinities to estrogen receptors isoformes. Summary of the data for ERα, ERβ or ERβ2 prepared from specifically transfected cells [Kuiper *et al.*, 1997, 1998; Petersen *et al*., 1998]. The range of values obtained irrespectively of techniques used is given in parentheses.

Competitor	Relative binding affinity [%]			Inhibition constant [nmol/L]		
	ERα	ERβ	ERβ2*	ERα	ERβ	ERβ2
17β-estradiol	100	100	100	0.13	(0.12 - 0.17)	3.02
Coumestrol	(20 - 94)	(100 - 185)	39	0.14	(0.07 - 0.11)	7.7
Genistein	(0.7 - 5)	(13 - 65)	7	2.6	(0.26 - 0.3)	42
Daidzein	(0.1 - 0.2)	0.5				
Biochanin A	<0.001	<0.001				
Formononetin	<0.001	<0.001				

* Calculated by authors from original data

The effect of genistein binding to ERβ is considered as partial agonistic due to its ability to bind receptor in the same place as natural estrogens but changing the orientation of helix 12 within AF-2 functional domain in a similar way as ER antagonists [Pike *et al.*, 1999]. The derivative of genistein, 6-carboxymethylated genistein, has been also tested for estrogenic activity. Its effects on ERs differ from that of genistein and were classified as mixed agonist (on ERβ) and antagonist (on ERα) with unique effects on the vasculature, bone and uterus [Somjen *et al.*, 2002]. The other potent phytoestrogen present in grapes and wine is the resveratrol (trans-3,5,4'-trihydroxystilbene). It has a differential effect on ERα and ERβ in an ERE sequence-dependent manner and therefore, was classified as mixed agonist (on ERβ) and antagonist (on ERα) [Bowers *et al.*, 2000]. Also the 8-prenylnaringenin, extracted from hops and present in beer, has similar high affinity to both, hERα and hERβ, found to be greater than that of coumestrol or genistein [Milligan *et al.*, 2002; Takamura-Enya *et al.*, 2003].

METHODS FOR DETERMINATION OF LIGAND EFFECTS ON ESTROGEN RECEPTORS

There is no one universal method for determining the potency of estrogenic ligands. The reason for this is that in mammals there exist many different isotypes of ERs and the different mechanisms of intracellular action cause that there is no one universal method for determining the potency of estrogenic ligands. Different methods give in most cases additional information which usually remains to be verified under *in vivo* conditions. In attempts to optimise the detection system, an interesting model for integrated evaluation of estrogenic activity has been developed in the form of ER action indicator (ERIN) transgenic mouse [Nagel *et al.*, 2001]. Although it integrates the upstream requirements in ER action, including the receptor, ligand, and accessory comodulators, more data are needed before results obtained can be properly interpreted. The use of cell culture or cell suspension is another alternative and many different cell lines with recognised expression of ERs isoformes are now commercially available. The problem in that case is that the results usually vary due to dynamic changes occurring within cell life cycles [Snochowski & Wolinska-Witort, 2001]. The most frequently used is the well-established ER-ligand binding assay for the relative binding affinity (RBA) using uterine cytosolic receptor preparation [*e.g.* Shi *et al.*, 2001; Branham *et al.*, 2002]. A more complex evaluation has been suggested as *e.g.* combination of *in vitro* and *in vivo* assays (competitive binding with the mouse uterine ER, transcriptional activation in HeLa cells transfected with plasmids containing ER and its responsive element, and the uterotropic assay in mice) [Shelby *et al.*, 1996].

The biological activity ranking of different exogenous ligands to ER showed a wide range of a 6 orders of magnitude spread of RBAs [Shi *et al.*, 2001; Branham *et al.*, 2002]. However, the prediction of biological potency, especially in the case of weak ligands, requires a detailed recognition of the way that may interfere with the mechanism of endogenous estrogens action.

The increasing knowledge of the effect of ligand on receptor structure-function relationship resulted in a number of methods suitable for *in vitro* estimation of *e.g.*: coactivator recruitment [Liu *et al.*, 2003]; nuclear receptor corepressor binding, a model system proposed [Webb *et al.*, 2003]; co-activator SRC1 nuclear receptor-binding domain, yeast two-hybrid detection system [Lee *et al.*, 2002]; conformational changes within LBD of ER using designer binding proteins (yeast two-hybrid techniques) [Koide *et al.*, 2002]; target promoter regulation [Hall & Korach 2002]; hERα-ERE binding screening assay [Kim *et al.*, 2003].

Furthermore, the high resolution online HPLC-MS biochemical detection system has been developed to screen plant natural product extracts for ERα and ERβ binding activity [Schobel *et al.*, 2001]. Different commercial kits are also available for screening ERs ligands, as well as, for their effects on ERα coactivator binding using products of ERα or ERβ isolated from specifically transfected cells.

EVALUATION OF PHYTOESTROGENS AT TISSUE LEVEL

Tissue regulatory level is understood as local hormonal environment that secures its normal physiological function. It includes a paracrine and intracrine action of estrogens produced in gonadal and many extragonadal tissues as the brain, breast, bone or vasculature. It was recognized that the aromatase, the key enzyme in estrogen synthesis, is locally expressed under the control of tissue-specific promoters and transcription factors and may generate high levels of estradiol without significantly affecting circulating levels [Labrie, 2003; Simpson *et al.*, 2002]. Therefore, the activity of ER in the particular tissue depends not only on its subtype specific expression but also on local hormonal environment. The use of receptor soluble fraction separated from tissue together with endogenous hormones for ligand affinity assay gives specific information often described as functional receptor activity. For more detailed description

we have selected the uterus as the most common target for screening estrogenic activity of xenobiotics, the pituitary as a key gland in endocrine regulations, and the thyroid as tissue also recommended for screening effects of endocrine disruptors [Goldman *et al.*, 2000]. Although the endocrine function of ERs in the pituitary and uterus is relatively well recognized, their role in thyroid gland is the least understood. The initial suggestion that thyroid can be directly responding to phytoestrogens has been based on indirect observations [Mawson *et al.*, 1994; Watanabe *et al.*, 2000; Horn-Ross *et al.*, 2002]. The mechanism responsible for clinical response was found to be other than well-known inhibition of thyroid peroxidase (TPO) catalysed reactions by phytoestrogens [Doerge, 2002], and therefore, the involvement of ER might be considered.

Animals. Different model animals have been used to produce data applicable for healthy man due to limited access to its tissue sample. The most common experimental animals are female rodents subjected to ovariectomy in order to avoid endocrine changes associated with estrus cycle. However, the hormonal changes after removal of ovaries differ with time from operation as well as *e.g.* ER activity in the uterus. The seasonally breeding animals may serve as a unique physiological model when used at the time of anoestrous period characterized by temporary stabilized hormonal activity along the hypothalamo--pituitary-ovarian axes [Snochowski, 2002]. Therefore, we have selected matured anoestrous ewes for the study of ER at tissue level. These animals have intact ovaries important for ER activities, which changes after ovariectomy performed also at the time of anoestrous [Snochowski *et al.*, 2002]. The size of ovine tissues collected from one animal is usually sufficient for analytical use. The tissue samples can be colleted directly in the slaughterhouse within the shortest possible time and when immediately frozen in liquid nitrogen may be stored at -80°C for long time until analysis.

Preparation of receptor fraction. Preparation of cell free tissue extracts is the procedure integrating ERs from different cell types and its results highly depend on conditions used already at the time of homogenisation. This is due to differential distribution of ER types within intracellular compartments and dynamic ER shuttling to cytoplasm [Maruvada *et al.*, 2003], as well as, the fact that ER binding to nuclear structures differs in strength and can be fractionally solubilized by increasing the concentration of ions [Boyer *et al.*, 2000]. Furthermore, in addition to the known effect of temperature on partitioning of ER to soluble fraction [Puca *et al.*, 1986] and the stabilizing effect of molybdate ions on ER [Skipper *et al.*, 1985] the presence of divalent metal ions at nanomolar concentration may block the ligand binding to ER [Martin *et al.*, 2003]. Therefore, the time from homogenizing to separation of solubilized receptor, as well as, buffer composition and the temperature are initial determinants of the quality of receptor fraction.

Example. In order to distinguish the soluble and structurally bound ER fraction we have used the following procedure: Tissue samples (0.5 to 1 g) were ground under liquid nitrogen to fine powder, suspended in 3 to 5 mL of ice-cold assay buffer (10 mmol/L Tris-HCl pH 7.4, 1.5 mmol/L EDTA, 1 mmol/L sodium thioglycolate, 10 mmol/L sodium molybdate, 10% glycerol) and centrifuged for 60 min at 100 000 x g. The collected supernatant was used for analysis as soluble receptor fraction. The resulting pellets were suspended 1:2 (w/v) with buffer containing 0.6 mol/L NaCl, incubated for 1 h at 0-4°C and after adding of equal volume of assay buffer (without NaCl) were centrifuged under similar conditions. The obtained supernatant was used for analysis as salt extractable (structurally bound) receptor fraction.

Blood contamination. The amount of blood contaminating the soluble receptor fraction is usually low and can be determined by direct photometric measurement as described by Dahlberg [1983]. This may be of importance especially to species with high blood circulatory level of steroid binding proteins. It was shown that human sex hormone-binding globulin binds directly not only endogenous steroids but also phytoestrogens [Jury *et al.*, 2000].

Enzymatic activity. The number of steroid transforming enzymes can be present in the receptor containing soluble fraction. The most important seem to be enzymes of aldo--keto reductase superfamily (AKR1C subfamily) 3-keto-, 17-keto-, and 20-ketosteroid reductases as well as 3α-, 17β-, and 20α-hydroxysteroid oxidases function as regulators converting potent sex hormones (androgens, estrogens, and progestins) into their cognate inactive metabolites or *vice versa* [Rizner *et al.*, 2003; Snochowski, 2003]. For example, the conversion of 3α-androstandiol to the most potent androgen 5α-dihydrotestosterone takes place in the prostate [Rizner *et al.*, 2003] in contrast to skeletal muscle where reverse conversion practically inactivates 5α-dihydrotestosterone hormonal activity [Snochowski *et al.*, 1986]. Phytoestrogens can be also exposed to enzymatic conversion, especially when microsomal fraction was not removed from receptor preparation. It was shown on human liver microsomes that genistein is a substrate for cytochrom P450 enzymes producing 3'-OH-genistein as the main metabolite [Hu *et al.*, 2003]. Therefore, the enzymatic conversion should be considered when interpreting data obtained form receptor fractions extracted from tissues.

Endogenous steroids. The amounts of endogenous steroids in the isolated receptor fraction represent the pool of hormones present within the tissue at the time of collection. Binding data obtained from such preparation may be considered more informative in regard to tissue specific hormonal milieu. However, the steroid free fraction can be prepared using simple passage through the Lipidex 1000 column [Dahlberg *et al.*, 1980] which also efficiently absorbs free thyroid hormones [Li *et al.*, 1991] and does not exhibit binding properties for soluble proteins at 0° and 37°C [Glatz *et al.*, 1983].

Hydrophobic micelles. The hydrophobic substances released from tissue during homogenisation process usually form a distinguished layer on the top, which can be simply discarded. However, in specific cases they may form hydrophobic micelles evenly distributed within the supernatant and cannot be mechanically removed.

The direct use of such heterogeneous steroid receptor fraction prepared from porcine seminal vesicles resulted in false positive cooperativity ligand binding effect that completely disappeared after delipidation on Lipidex 1000 [Dahlberg *et al.*, 1980]. Similar effects have been observed in ER soluble fraction prepared from thyroid gland [Snochowski *et al.*, 2002]. This may be due to unique function of thyroid gland with structure adapted for synthesis of thyroglobulin that undergoes specifically controlled intracellular trafficking [Marino & McCluskey, 2000] including the formation of multimerized gigantic globules stored in the lumen of follicle [Berndorfer *et al.*, 1996].

Example. Delipidation of soluble receptor fraction obtained from thyroid gland was performed according to previously described method recommended for removal of hydrophobic compounds from biological fluids [Dahlberg *et al.*, 1980]. Lipidex 1000 (Packard Instrument Co., Downers Grove, IL, USA) initially suspended in methanol was transferred to columns (3 x 0.5 cm) and introduced to water phase by stepwise washing with diminishing methanol concentrations (75, 50, 25, 0%). Prior to the delipidation all columns were equilibrated with assay buffer at low temperature (0-4°C). After sample passage the first and last part colleted were discarded in order to avoid dilution effect of preparation used for receptor analysis. The effect of delipidation on ^{3}H-17β-estradiol receptor binding, evaluated according to Scatchard, is presented in Figure 1.

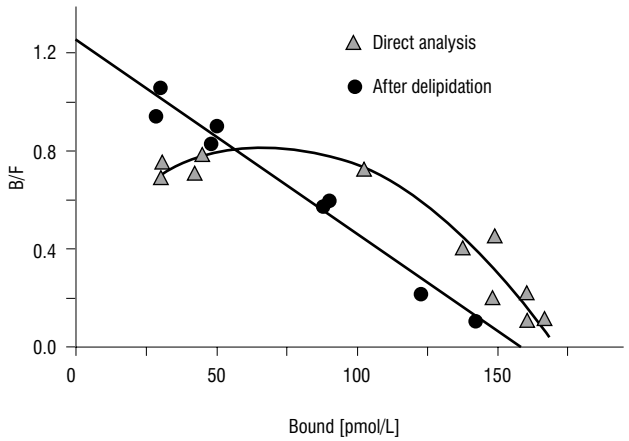

FIGURE 1. Comparison of Scatchard plots obtained for ovine thyroid estrogen receptor analysed directly in the soluble fraction or after delipidation on Lipidex 1000.

Receptor binding assay. Demonstration of single class of high affinity binding sites for each newly prepared receptor fraction is a part of validation of ligand affinity assay. This can be performed using receptor binding assay, a typical saturation analysis, where constant amount of receptor is incubated in the presence of different concentrations of ligand. The most suitable labeller allowing to measure reaction product at femtomolar level seems to be ^{3}H-17β--estradiol since other forms may affect its affinity to ER as in case of 16α-[125I]-iodo-17β-estradiol [Kuiper *et al.*, 1998]. The use of ^{3}H-moxestrol (R2858) is not recommended because its binding affects ER structure in a way, which slightly differs from that of estradiol [Barkhem *et al.*, 2002]. The optimal range of ligand concentrations resulting in evenly distributed binding data can be calculated from expected dissociation constant. When two times dilution steps are used, the following equation describes the highest concentration of ligand:

$$T_{max} = Kd \times 2^{0.5(n-1)} \text{ [mol/L]} \quad (1)$$

where Tmax is the highest ligand concentration to be used [mol/L]; Kd is dissociation constant [mol/L]; "n" is the number of consecutive two times diluted solutions [Snochowski, 1985].

The time of incubation sufficient to achieve equilibrium for ER-ligand interaction at low (0-4°C) is about 16 h. The shortening of time by elevating temperature may have significant effect on ligand interaction with other proteins (enzymes) and its use should be separately verified.

The separation of ER bound fraction can be performed using different techniques including solid phase absorption and gel filtration. The best separation is achieved when all free and nonreceptor bound ligand is removed without affecting ER-ligand complex. The level of non-specific binding should be determined by parallel incubations containing addition the receptor saturable excess of unlabelled estradiol. Instead of estradiol the diethylstilbestrol can be used as a receptor specific displacer because it practically not reacts with blood binding proteins; its RBA to sex hormone-binding globulin is less then 0.3% [Hodgert Jury *et al.*, 2000]. The quantitative measurements of samples from the assay should be preformed in a way allowing their reliable expression in molar units.

Calculations. The calculation of receptor binding data is included in different computer programs allowing alternative use of different methods for determination of ligand affinity and receptor binding capacity [Hulme & Birdsall, 1992; Munson & Rodbard, 1980; Snochowski, 1985; Wells, 1992]. The receptor preparation suitable for ligand affinity assay should be characterized by single type of receptor binding sites (Hill coefficient close to 1) and high ligand affinity (Kd <10 nmol/L). Although for good receptor preparations the dissociation constant calculated from nonlinear (Langmuir binding isotherm) or linear transformation (Schatchard equation) do not differ, the later method seems to be more suitable allowing to use appropriate statistics for linear regression data as quality criteria.

The receptor binding capacity initially calculated is expressed as molar concentration of binding sites present in the incubation mixture. This allows direct estimation of optimal dilution of receptor fraction for its subsequent use in the ligand binding assay. The simplest expression of binding data enabling tissue comparison is its relation to weight of tissue (average density). The complementary expression of data related to DNA unit is highly recommended because the average size of the cell may differ even within the same type of tissue depending of its actual activity. The amounts of DNA can be either determined in homogenate or in the pellets obtained after separation of receptor fraction. Moreover, when binding data are expressed in units of fmol/mg DNA the average cellular content of receptor ligand binding sites can be simply calculated by multiplication with conversion factor F=3.7338 calculated using Avogadro constant and fixed DNA content in the mammalian cell assumed to be 6.2 pg [Enesco & Leblond, 1962].

TABLE 2. Comparison of tissue estrogen receptors analysed in in the samples collected from three anoestrous ewes (means ± SD). The binding capacity was determined by ligand binding assay performed in the soluble and structural (NaCl extractible) fraction. Data were calculated according to Scatchard and statistical comparison was performed using Kruskal-Wallis test followed by Mann-Whitney's U test with the help of the program Statistica for Windows (Statistica™ PL).

Tissue	DNA	Dissociation	Binding capacity [fmol/g]		Total receptor binding sites		
	[mg/g of tissue]	constant [nmol/L]	Soluble fraction	NaCl extracted	fmol per mg DNA	N x 10^3 per cell	Per cent extracted
Pituitary	1.70 ± 0.72[a]	0.58 ± 0.08[a]	2500 ± 260	410 ± 97[a]	1840 ± 560[a]	6.9 ± 2.1[a]	14 ± 3.3[a]
Uterus	3.00 ± 1.28[b]	0.72 ± 0.41[a]	2400 ± 390	590 ± 96[a]	906 ± 216[b]	3.4 ± 0.8[b]	20 ± 2.7[b]
Thyroid*	4.91 ± 0.95[c]	0.14 ± 0.07[b]	2100 ± 430	121 ± 27[b]	450 ± 120[c]	1.7 ± 0.4[c]	5.4 ± 1.2[c]

* - soluble receptor fraction was analysed after delipidation with Lipidex 1000. Different letters indicate significant differences ($p < 0.05$) within the columns.

Example. Portions of 0.1 mL of receptor fraction were incubated in duplicates with 0.1 mL of assay buffer containing six consecutive dilutions of radioligand at final concentrations ranging from 0.05 to 1.6 nmol/L of ^{3}H-17β-estradiol (3.37 TBq/mmol; Amersham Pharmacia Biotech, Little Chalfont, UK) during 16 to 20 h at 0 to 4°C. The bound fraction was separated after 20-min incubation with 0.5 mL of ice cold dextran couted charcoal (0.5% Norit A, Sigma-Aldrich Corp. St. Louis, MO, USA; 0.05% dextran T 70, Pharmacia, Uppsala, Sweden) following 20-min centrifugation at 1 000 x g). The aliquots of 0.5 mL of supernatant were transferred to 10 mL scintillation vials containing dioxane scintillation cocktail, and the radioactivity was measured using liquid scyntilation counter (Beckman LC 6000 TA). The nonspecific binding was determined in parallel incubations containing 200 times excess of diethylstilbestrol (Sigma, St. Louis, MO). The receptor binding capacity (Bmax) and dissociation constant (Kd) were calculated according to Scatchard from plots with slope different from zero with 95% confidence [Snochowski, 1985]. The estimated amounts of receptor ligand binding sites were expressed in relation to gram of tissue wet weight, and the average cellular content was calculated using the data from analysis of DNA performed in tissue pellets according to the method of Burton as described previously [Dahlberg *et al.* 1981].

The data obtained from analysis of pituitary, uterine and thyroid receptor are summarized in Table 2. Each tissue was characterized by the presence of single class of high affinity, low capacity, binding sites for estrogens. The dissociation constants for ER from pituitary and uterus were similar and significantly higher then those from thyroid gland. This may be an effect of endogenous steroids that were removed from thyroid preparation during the passage through Lipidex column. The concentrations of ER binding sites in soluble fractions expressed in relation to tissue weight were similar in all three tissues. However, they did differ in amounts localized in NaCl soluble fraction, especially when expressed as percentage of total number of binding sites. The DNA analysis revealed that all three tissues contained different densities of cells being the lowest in the pituitary and the highest in the thyroid. Consequently, the binding data expressed in relation to DNA or as the average cell content have shown the highest number of binding sites in the pituitary, lower in uterus and the lowest in thyroid gland. The amounts of receptor in the NaCl extractible fraction varied in average between 5 and 20 percent in tissue specific manner. They may represent significant population of specific type of the receptor that cannot be distinguished by ligand binding analysis. The presented data indicate that the ovine ER from the pituitary, uterus, as well as, from thyroid has a typical ligand binding characteristics described for other mammals. The relatively low individual variations of binding parameters presented in Table 2 imply that the ER activity is temporary stabilized at the time of seasonal anoestrous.

Ligand affinity assay. The ligand affinity assay preformed using tissue extracted receptor fraction is often described as "functional" receptor assay to emphasise that the results obtained reflect the resultant effect of ligand interaction with actual mixture of ERs isoformes deriving from different types of the cells. In the traditional radioligand binding assay, the constant amounts of receptor and radioligand is incubated in the presence of different amounts of tested compound under incubation conditions essentially the same as those described for ligand binding receptor assay. The optimal concentration of radioligand is determined by its affinity to the receptor, and the concentration exciding two times Kd value is considered as sufficient for ligand displacement study. Furthermore, the amount of radioligand specifically bound to receptor in the absence of competitor should be sufficient for reliable measurement in hundred times lower quantities. The use of different solvents is often necessary to introduce hydrophobic ligands into incubation medium at concentrations often reaching micromolar level. When dimethylsulfoxide, dioxane, methanol, ethanol or other solvents are used they should be included to all reference samples in order to avoid problems with their possible effects on receptor properties, as well as, on separation of free and receptor bound fraction. In order to secure reaction conditions closer to equilibrium, the use of longer time of incubation, usually lasting about 24 h, is recommended.

Calculation. The data obtained from each series of samples containing different concentration of tested compound are used to calculate the concentration resulting in the 50% displacement of ^{3}H-estradiol (radioligand) from receptor binding sites (IC_{50}). This can be achieved using *e.g.* nonlinear four-parameter logistic model [Schults *et al.*, 1988] or linear log/logit transformation [Rodbard & Rayford, 1968]. The relative binding affinity (RBA) is then calculated as the ratio of IC_{50} values obtained for standard agonist (estradiol) and for competitor. The RBA value for estradiol is often set arbitrary to be equal 100 data for tested compounds are presented as its percentage.

The equilibrium inhibition constant (Ki) can be calculated from the equation:

$$Ki = IC_{50}/[1 - (L/Kd)]; \quad (2)$$

where IC_{50} is the calculated concentration of inhibitor, L is the concentration and Kd is the dissociation constant of the radioligand [Cheng & Prusoff, 1973]. For specific cases, when the slope function for competitor differs from 1, more accurate equations can be used as proposed by Cheng [2001].

Example. The ligand binding affinities of ER to estrone, estriol, testosterone, cortisol, genistein, daidzein (Sigma, St. Louis, MO) and coumestrol (Eastman Kodak, Rochester, NY) were determined using 17β-estradiol (Sigma, St. Louis, MO) as reference and ^{3}H-17β-estradiol (3.37 TBq/mmol) as labeller at final concentration of 6.2 nmol/L. The analysis was performed by 24-h incubation in water/ice bath of series of duplicated tubes containing 0.05 mL of ^{3}H-estradiol, 0.05 mL of competitors and 0.1 mL of soluble receptor fraction. The series of two or ten fold consecutive dilutions of displacer ranged at final concentration from 0.4 to 27 nmol/L or from 0.7 to 700 nmol/L for estradiol or other competitors, respectively. The nonspecific binding was determined by parallel incubations using labeler solution containing a 100-fold excess of non-radioactive diethylstilbestrol (Sigma, St. Louis, MO). The separation of bound fraction and radioactivity measurement was performed as described in the part for receptor analysis. The data were evaluated using log/logit plots (Figure 2) to estimate the IC_{50} value (the concentration of competitor at half-maximal specific binding). The RBA values were expressed in relation to estradiol arbitrary set to 100. The inhibition constants (Ki) were calculated using the Cheng-Prusoff equation [Cheng & Prusoff, 1973].

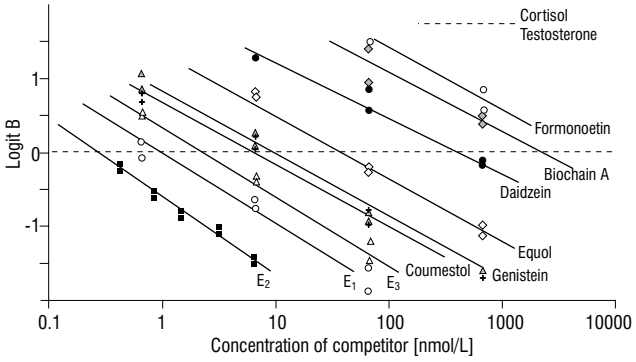

FIGURE 2. The example of ligand affinity analysis of dietary phytoestrogens to estrogen receptor from ovine pituitary. E_1, E_2, and E_3 indicates the effect of are natural estrogens, estrone, estradiol, and estriol, respectively.

The results of RBA and Ki values obtained for soluble ER from ovine pituitary, uterus and thyroid are presented in Table 3. The binding potency for steroid hormones in all thee tissues have shown a typical order for mammalian ER, estradiol> estrone> estriol, without significant influence of testosterone or cortisol. The RBA values for phyto-estrogens formed similar order of potency, as that described for pure ER isoformes (see Table 1) with the exception of coumestrol showing affinity similar to that of genistein. Despite these similarities, the affinities of phytoestrogens were much higher to ER from pituitary and thyroid than from uterus.

TABLE 3. Characteristics of ligand binding specificity of ovine estrogen receptors prepared from pituitary, uterus and thyroid. The relative binding affinities were calculated from log/logit plots and expressed as percentage of molar concentration ratios of estradiol and competitor resulting in 50% displacement of 3H-estradiol from receptor binding sites. Inhibition constants were calculated from IC_{50} values using the Cheng-Prusoff equation.

Competitor	Relative binding affinity [%]			Inhibition constant [nmol/L]		
	Pituitary	Uterus	Thyroid[1]	Pituitary	Uterus	Thyroid[1]
Oestradiol	100	100	100	0.20	0.22	0.03
Estrone	42	18	28	0.47	1.2	0.12
Estriol	11	10	19	1.8	2.2	0.17
Coumestrol	5.3	0.8	5.4	3.7	28	0.61
Genistein	4.9	0.8	7.5	4.0	28	0.44
Equol	0.4	0.3	ND	49	74	ND
Daidzein	0.07	0.04	0.08	280	560	42
Biochanin A	0.003	<0.0001	ND	6600	$>10^4$	ND
Formononetin	<0.002	<0.0001	ND	>9000	$>10^4$	ND
Testosterone	<0.0001	<0.0001	<0.0001	$>10^4$	$>10^4$	$>10^4$
Cortisol	<0.0001	<0.0001	<0.0001	$>10^4$	$>10^4$	$>10^4$

ND - not determined, [1] analysed using receptor fraction after delipidation on Lipidex 1000.

The calculated inhibition constants have shown a similar pattern of values to RBA's, although the numbers for thyroid ER were one order of magnitude lower compared to pituitary, as a consequence of removal of endogenous steroids during delepidation. The presented data indicate that expected concentration of genistein or coumestrol affecting ER in the pituitary is on the level of 5 nmol/L, while for uterine ER response is at least five times higher. The effective concentrations of these phytoestrogens in the thyroid can be estimated only on the basis of RBA values, and seem to be similar to those calculated for pituitary gland.

CLINICAL IMPLICATIONS OF PHYTOESTROGEN ACTION ON ESTROGEN RECEPTORS

The recent progress in steroid receptor research has substatial influence on clinical understanding of ER action [Speroff, 2000]. It is expected that the knowledge on molecular effects of phytoestrogens on ERs should help to create the bases for their use as alternative or complementary therapy in women and men [Arena *et al.*, 2002; Burton & Wells, 2002; Wilkinson & Chodak, 2003].

The clinical and pharmacokinetical studies have shown that in healthy man the consumption of aglycone form of soy isoflavones (up to 16 mg/kg BW; 90% of genistein) had no clinically significant behavioural or physical effects, and calculated elimination half-lives for free and total genistein was 3.2 and 9.2 h, respectively [Busby *et al.*, 2002]. The similar results were reported for postmenopausal women suggesting that chronic dosing at 12-24-h intervals would not lead to progressive accumulation of these isoflavones [Bloedon *et al.*, 2002]. The tissue distribution studied on rats has shown that genistein given in a diet was accumulated exclusively as aglycone in brain, liver, mammary, ovary, prostate, testis, thyroid and uterus (up to 7 nmol/g in liver) while in serum was present in above 95% as conjugate [Chang *et al.*, 2000]. In the study on pregnant rats, it was shown that dietary phytoestrogens may cross the placenta and significant concentrations of genistein were

detected not only in foetal blood but also in the brain [Doerge *et al.*, 2001]. In human, daidzein and genistein have been found at nanogram quantities in samples of second trimester amniotic fluid indicating direct exposure of foetus to dietary isoflavones [Foster *et al.*, 2002].

The biotransformation of dietary phytoestrogens may change the estrogenic potency of consumed phytochemicals. The most spectacular example is the conversion of daidzein to equol taking place within the intestinal bacterial metabolism. The reaction product, equol, has about ten times higher affinity to ERs then daidzein (see below). Setchell *et al.* [2002] have presented evidence for existence of two distinct subpopulations of people differing in the ability to make equol. The determination of urinary phytoestrogens has been suggested as dietary biomarker useful for establishing healthier dietary patterns [Lampe, 2003].

Thus, phytoestrogens can be broadly distributed among all human tissues and may reach the significant concentration for ER interaction. However, in the recent review the worrying states that before recommendations regarding phytoestrogen supplements can be safely made, we must have more information on the effects of the extracts on bone, heart and breast health [Kurzer, 2003].

Dietary consumption of genistein and daidzein is also associated with a decreased breast cancer risk that may arise in part from the suboptimal configuration induced in the transactivation helix of ERβ [Wiseman & Duffy 2001]. In the study performed on human breast cancer cells (MCF--7; ERα, ERβ), genistein stimulated growth and proliferation at low concentrations but inhibited it at high concentrations. Nevertheless, in the evaluation of the general effect of phytoestrogens on breast cancer, it is concluded, "if breast cancer patients enjoy soy products, it seems reasonable for them to continue to use them" [Messina & Loprinzi, 2001]. In the recent review dedicated to different toxicological aspects of phytoestrogens action, the similar conclusion has been drawn, that the current literature supports the safety of isoflavones as typically consumed in diets based on soy or soy-containing products [Munro *et al.*, 2003]. Thus, the general statement made by Jordan *et al.* [2001] that the development of selective estrogen receptor modulators might result in the introduction of new multifunctional medicines with applications as preventive agents or treatments cancer (breast, endometrial), coronary heart disease, and osteoporosis, may concern also phytoestrogens.

SUMMARY

The currently available data fully confirm that dominant effects of phytoetrogens in mammals are caused by their direct interaction with ERs. However, the existence of functionally different multiple forms of ERs expressed in tissue and cell specific manner do not allow for the use of a single detection system for evaluation of phytoestrogens activity. The complementary description of the effect of phytoestrogen on ER can be achieved by measurement binding affinity to (1) specific ER type or its isoform including further determination of the effect of ligand on receptor affinities to co-factors and EREs; (2) tissue ER containing extracts, which allows to assess the effective concentration of ligand at local hormonal environment. The results from ER specific type of analysis refers to single cell response and gives qualitative information on the ligand activated mechanism of ER action. The data obtained from ER containing extracts characterize tissue specific responses and allow assessing the biologically effective concentrations of phytoestrogens. It is expected that the ongoing research on identification of ER inducible genes in vertebrate genomes may result in a more complex evaluation of estrogenic responses using proteins whose function is directly affected by phytoestrogens [Bajic *et al.*, 2003].

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