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COMMENTARY

Dietary Soy-Derived Isoflavone Phytoestrogens

COULD THEY HAVE A ROLE IN CORONARY HEART DISEASE PREVENTION?

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ABSTRACT. Soy protein-containing foods are a rich source of isoflavone phytoestrogens, such as genistein and daidzein. There is great interest in these substances, as lower rates of chronic diseases, including coronary heart disease, have been associated with high dietary intake of soy-containing foods. Soy phytoestrogens bind weakly to estrogen receptors, and some bind more strongly to estrogen receptor-β compared with estrogen receptor-α. A meta-analysis has indicated that isoflavone phytoestrogens lowered plasma cholesterol concentrations in subjects with initially elevated levels, but had little effect in subjects with normal cholesterol concentrations. These substances reportedly may also have beneficial effects on arterial endothelial function. In addition to these potentially antiatherogenic effects, many laboratories are investigating other possible mechanisms, including antioxidative and antiproliferative properties of these substances. We have shown that dietary supplementation with soy-derived isoflavones reduced the *in vitro* oxidation susceptibility of low-density lipoprotein (LDL). To further explore this phenomenon, we incorporated genistein and daidzein into LDL molecules *in vitro* with the aid of an artificial transfer system. However, it was necessary to convert the isoflavone molecules to fat-soluble derivatives, fatty acid esters (analogous to esterified endogenous estrogens, which are known to occur *in vivo*), to achieve significant incorporation. The LDLs containing esterified isoflavones were shown to be less susceptible to oxidation *in vitro* than native LDL. We also employed U937 cell cultures for investigating the effects of isoflavone-containing LDLs on cell proliferation. Some of these LDLs exhibited antiproliferative effects in cultured U937 cells. In summary, lipophilic phytoestrogen derivatives could be incorporated into LDLs, increasing their oxidation resistance and antiproliferative efficacy *ex vivo*, both of which are, in theory, antiatherogenic effects. Further studies are needed to assess to what extent analogous effects could be produced *in vivo* and whether such substances have a role in hormone replacement and coronary heart disease prevention in postmenopausal women. BIOCHEM PHARMACOL **60**;1:1–5, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. genistein; daidzein; LDL; estrogen receptors; antiatherogenesis; estrogen replacement

The abundance of certain isoflavone phytoestrogens in Asian diets and the lower rates of "Western" diseases such as coronary heart disease, as well as breast, prostatic, and colon cancers in Asian populations have suggested a protective role for these mostly soy-derived substances [1, 2]. The possibility that phytoestrogen intake has an antiatherogenic effect has received support from the existence of plausible underlying mechanisms of protection, such as plasma lipid risk factor modification, antioxidant protection of LDLs,† and antiproliferative as well as vascular reactivity effects.

ESTROGENICITY OF SOY-DERIVED ISOFLAVONES

The cloning and description of a novel estrogen receptor, ER-β [3], has made it possible to understand the selective effects of structurally closely related estrogenic substances. Although the exact physiological role of ER-β is still under investigation, tissue-specific differences in its expression provide explanations for differing effects of estrogenic substances in various tissues. For example, ER-β is expressed in non-reproductive tissues, such as bone and the vascular system [3, 4].

The primary soy-derived isoflavones genistein and daidzein both bind to ERs, a finding probably explained by their structural similarity with estrogens (Fig. 1). According to Kuiper *et al.* [5], the binding affinity of genistein to the recently discovered ER-β was about 20 times greater than to ER-α. Compared with estradiol, the binding affinity of genistein for ER-α was 4%, and for ER-β was 87% [5]. Compared with estradiol, the binding affinity of daidzein for ER-α and ER-β was 0.1 and 0.5%, respectively [5].

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† *Abbreviations*: LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; HDL, high-density lipoprotein; ER, estrogen receptor; and SERM, selective estrogen receptor modulator.

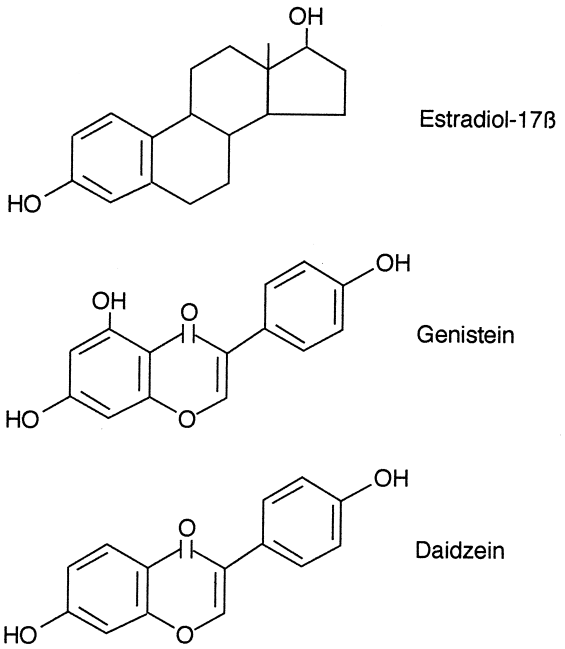

FIG. 1. Molecular structures of estradiol-17β, genistein, and daidzein.

Some experimental evidence obtained in animal models suggests that phytoestrogens may act as estrogen agonists under estrogen-depleted circumstances but exert antagonist activity under circumstances of estrogen surplus [6, 7]. Thus, the effect of isoflavone phytoestrogens may depend on both the estrogenicity of the surrounding milieu and the expression of ER subtypes.

MODIFICATION OF PLASMA LIPIDS AND LIPOPROTEINS

The serum cholesterol and LDL–cholesterol-lowering effects of soy protein-containing foods have been investigated in a large number of studies, which have been subjected to a meta-analysis [8]. This meta-analysis included 38 clinical studies reported in 29 articles. In summary, a mean intake of 47 g of soy protein daily was associated with a net reduction in serum LDL–cholesterol of 12.9%. However, the decrease was directly related to the initial serum cholesterol level, with insignificant changes in individuals with low initial cholesterol (−3.3% in the lowest quartile) and marked reductions in those with the highest levels (−19.6% in the highest quartile) [8]. Setchell [9] was the first to suggest that phytoestrogens contained in soy protein foods could contribute to the cholesterol-lowering effect, since they have weak estrogenic activity. As these phytoestrogens (primarily genistein and daidzein) bind to ERs, this is plausible. According to this theory, phytoestrogens would share the well-established LDL-receptor-inducing property of human estrogens. Evidence for this hypothesis was later produced in several studies carried out in animals (summarized in Ref. 10). Diets containing intact soy protein isolates (SP+) and soy protein isolates from which isoflavone phytoestrogens had been removed by ethanol extraction (SP−) were fed to non-human primates. Estrogen-like effects were observed in serum lipoproteins in SP+ animals showing reduced cholesterol (measured as VLDL plus LDL cholesterol) and elevated HDL cholesterol levels compared to SP− animals. In addition, the mean atherosclerotic plaque size was reduced in SP+ animals [11], suggesting the possibility of antiatherogenic effects caused by alterations in the serum lipoprotein profile.

EFFECTS ON VASCULAR REACTIVITY

In theory, phytoestrogens may also have direct effects on arterial walls, either through their inhibitory effect on vascular smooth muscle cell proliferation and migration [12, 13] or through an effect on vascular reactivity [14, 15]. The expression of ER-β in vascular and other non-reproductive tissues provides, at least in theory, one possible mechanism by which phytoestrogens could exert effects on the arterial system. Reduced smooth muscle cell proliferation may retard development of atherosclerotic plaques, and improved endothelial function may have vasodilatory effects resulting in an antiatherogenic effect. This has received support recently from studies carried out in male and female macaques with pre-existing diet-induced atherosclerosis [16]. These non-human primates received an SP+ or SP− diet for 6 months. Vascular reactivity was tested by giving an intravenous injection of acetylcholine in conjunction with angiography. In females, the SP− diet group was associated with a constriction of coronary arteries, whereas the SP+ group was associated with a vasodilatory response in the coronary arteries following acetylcholine injection. The response to acetylcholine was not significantly different in the SP+ and SP− groups in male macaques. Administration of intravenous genistein also improved vascular reactivity in female macaques in this study [16]. The intake of soy isoflavones was also shown to improve systemic arterial compliance in menopausal and perimenopausal women [17].

EFFECTS ON ATHEROSCLEROTIC PLAQUE FORMATION

The formation of atherosclerotic lesions is a combination of many processes. Generally, it is believed that lesion formation is initiated by a response to some endothelial injury brought about by hyperlipidemia, or by toxic or infectious agents. The cellular infiltration and proliferation caused by the injury then contribute to the formation of the advanced atherosclerotic lesion. Soy-derived isoflavone phytoestrogens have been shown to have antiproliferative properties *in vitro* [18, 19]. In particular, genistein is a specific inhibitor for tyrosine kinases [20]. Many growth factors influence cell proliferation through binding to cell surface receptors, resulting in activation of tyrosine kinase activity residing in the intracellular domains of the receptors, ultimately leading to cell division. Studies are in progress in

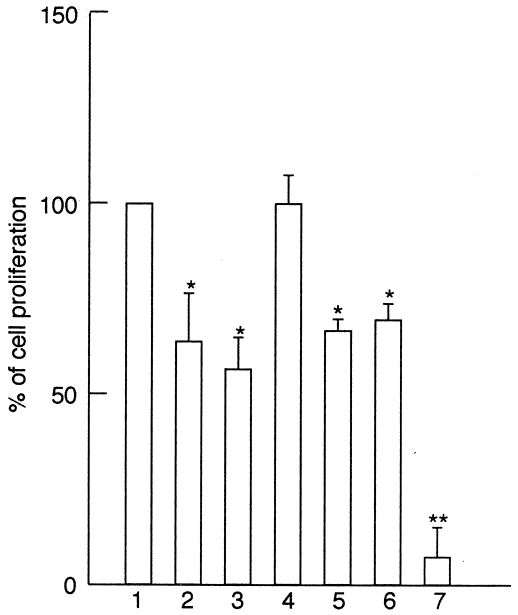

FIG. 2. Antiproliferative effects of isoflavone-containing LDL on U937 cells. LDLs (30 μg cholesterol/mL) containing different isoflavones were incubated with U937 cells for 22 hr at 37°. Cell proliferation was determined as [^{3}H]thymidine incorporation into DNA. All values (means ± SD) are expressed as a percentage of control LDL processed in the absence of isoflavones. 1: control; 2: genistein 7-oleate; 3: genistein 4′,7-dioleate; 4: daidzein 4′-oleate; 5: daidzein 7-oleate; 6: daidzein 4′,7-dioleate; and 7: daidzein 4′,7-dilinoleate. Key: (*) $P < 0.01$, and (*) $P < 0.001$ vs control. (Adapted from Ref. 21.)

several laboratories to better define the possible antiatherogenetic properties of genistein and other isoflavones and to determine whether the antiproliferative potency contributes to this. We set out to explore whether soy isoflavones could be incorporated into LDL particles and internalized via LDL receptors in cultured U937 cells [21]. This experimental setting was designed to investigate whether adequate numbers of isoflavone molecules could be delivered into cells to influence cell proliferation. Cultured U937 cells were used because their proliferation depends completely on an extracellular cholesterol supply, in this case provided by the addition of LDL particles to the medium [21]. We produced lipophilic fatty acid esters of genistein and daidzein and demonstrated that significant amounts could be incorporated into LDL. LDLs enriched with certain isoflavone esters significantly inhibited cell proliferation compared with native LDL (Fig. 2, data from Ref. 21). We were unable to incorporate significant amounts of free genistein or daidzein, which are relatively hydrophilic molecules, into LDL particles, and LDLs from these experiments did not influence cell proliferation. Endogenous human estrogens are present in blood and other tissues in esterified form [22]. Incubation of free estradiol with plasma produces a lipophilic estradiol derivative, which has all the properties of a fatty acid ester and can be incorporated into LDL, providing antioxidant protection for it [23, 24]. It is not clear whether isoflavones can form analogous lipophilic derivatives *in vivo*, and whether these substances could be introduced into lipoproteins. In theory, the entry of LDL-containing phytoestrogen derivatives with antioxidative and antiproliferative properties into the artery wall can be considered less atherogenic than the entry of native LDL.

ANTIOXIDANT ROLE OF ISOFLAVONE PHYTOESTROGENS

We were interested in yet another possible protective mechanism, which is the role of isoflavone phytoestrogens as antioxidants providing increased oxidation resistance for lipoproteins [25]. In a carefully designed study, we demonstrated that feeding of soy-derived isoflavones [genistein (36 mg daily) and daidzein (21 mg daily)] for 2 weeks resulted in increased oxidation resistance of LDL isolated at the end of the 2-week period. Purification of LDL from water-soluble contaminants ascertained that the altered oxidation susceptibility was due to alteration in the LDL particles themselves. In another study [26], we incorporated genistein and daidzein into LDL particles *in vitro* and investigated LDL oxidation resistance before and after incorporation. However, due to their relative hydrophilicity, these isoflavones were incorporated into LDL to only a small extent (0.33 molecule/LDL particle or less), and there was no change in the oxidation resistance of the particles. We then converted genistein and daidzein into fat-soluble derivatives by esterification with fatty acids to facilitate their incorporation into LDL. Oleic acid esters were incorporated most effectively, reaching a concentration of 2.19 molecules/LDL particle, or more, and resulted in significantly increased oxidation resistance *in vitro* [26]. On this basis, isoflavones may protect lipoproteins against oxidation, provided that they have been converted to lipophilic derivatives and incorporated into the lipoprotein particles. Although fatty acid esters of endogenous human steroids [22] including estradiol [27] are formed in the human organism and become incorporated in human LDL [23, 24, 28, 29], the existence of analogous phytoestrogen esters has not been reported.

ISOFLAVONE PHYTOESTROGENS: POSSIBLE ROLE IN POSTMENOPAUSAL HORMONE REPLACEMENT

A large number of observational studies have suggested that postmenopausal hormone replacement therapy (HRT) may, in addition to other benefits, also contribute to cardiovascular prevention [30]. The only large, randomized placebo-controlled intervention study involving HRT, however, did not show any benefit [31]. In the HERS study, increased thromboembolic complications were observed during the first year of the 4.1-year follow-up. This, and the fact that exposure to estrogen produces endometrial proliferation and a slightly increased breast cancer risk during prolonged use, have promoted the search for alternative hormonal therapies. A novel group of agents, the SERMs,

TABLE 1. Comparison between the effects of estrogen, SERM, and phytoestrogen

	Estrogen	SERM (1st generation)	SERM (2nd generation)	Isoflavone phytoestrogen
Uterus	Agonist	Partial Agonist	Antagonist	No effect
Breast	Agonist	Antagonist	Antagonist	Agonist/antagonist
Bone	Agonist	Agonist	Agonist	Agonist
Vasculature	Agonist	Agonist?	Agonist	Agonist
Lipids	Agonist	Agonist	Agonist	Agonist
Prototype substance	E_2-17β	Tamoxifen	Raloxifene	Genistein

are currently under intensive study. Raloxifene, a second generation SERM, appears to exert estrogen agonist effects in bone and the vascular system, while it exerts antagonist effects in endometrium and breast tissue [32, 33] (Table 1). In theory, phytoestrogens share properties with SERMs, suggesting that they could act as agonists in bone [34] and vascular tissues [16, 17], although the data are less clear-cut compared with SERM data [34, 35]. There are conflicting reports concerning their effect on breast tissue, with some experimental studies suggesting antagonist effects and others suggesting agonist effects [36, 37]. In one study using experimental animals, phytoestrogen administration did not cause endometrial hypertrophy [38]. However, extrapolation from human cell culture and animal data is questionable. The role of phytoestrogens as estrogen agonists and antagonists needs to be investigated in more human studies.

We are interested in the possibilities of enriching diets with natural substances, such as soy-derived phytoestrogens, presumed to have antiatherogenic properties. For example, we think that fat-soluble substances accumulating in LDL or otherwise producing stable alterations in the LDL structure are of interest. Such LDL particles would, in principle, preserve their resistance to atherogenic changes (e.g. lipid peroxidation) also after the LDL particles have been sequestered in the artery wall and thus have become isolated from water-soluble antioxidants present in the circulation. Further studies are needed to clarify the mechanisms underlying the finding of increased oxidation resistance of LDL following intake of soy-derived isoflavone phytoestrogens. The possibilities of percutaneous administration of phytoestrogen derivatives should also be explored.

References

1. Adlercreutz H and Mazur W, Phyto-oestrogens and western diseases. *Ann Med* **29:** 95–120, 1995.
2. Clarkson TB, Anthony MS and Hugher CL Jr, Estrogenic soybean isoflavones and chronic disease. Risks and benefits. *Trends Endocrinol Metab* **6:** 11–16, 1995.
3. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S and Gustafsson J-A, Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* **93:** 5925–5930, 1996.
4. Enmark E and Gustafsson J-A, Estrogen receptor β—a novel receptor opens up new possibilities for cancer diagnosis and treatment. *Endocr Related Cancer* **5:** 213–222, 1998.
5. Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B and Gustafsson J-Å, Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology* **139:** 4252–4263, 1998.
6. Messina M, Soy intake and cancer risk: A review of the *in vitro* and *in vivo* data. *Nutr Cancer* **21:** 113–131, 1994.
7. Mäkelä SI, Pylkkänen LH, Santti RSS and Adlercreutz H, Dietary soybean may be antiestrogenic in male mice. *J Nutr* **125:** 437–445, 1995.
8. Anderson JW, Johnstone BM and Cook-Newell ME, Meta-analysis of the effects of soy protein intake on serum lipids. *N Engl J Med* **333:** 276–282, 1995.
9. Setchell KDR, Naturally occurring non-steroidal estrogens of dietary origin. In: *Estrogens in the Environment II: Influences on Development* (Ed. McLachlan JA), pp. 69–85. Elsevier, New York, 1985.
10. Anthony MS, Clarkson TB and Williams JK, Effects of soy isoflavones on atherosclerosis: Potential mechanisms. *Am J Clin Nutr* **68**(Suppl)**:** 1390S–1393S, 1998.
11. Anthony MS, Clarkson TB, Bullock BC and Wagner JD, Soy protein versus soy phytoestrogens in the prevention of diet-induced coronary artery atherosclerosis of male cynomolgus monkeys. *Arterioscler Thromb Vasc Biol* **17:** 2524–2531, 1997.
12. Fujio Y, Yamada F, Takahashi K and Shibata N, Responses of smooth muscle cells to platelet-derived growth factor are inhibited by herbimycin-A tyrosine kinase inhibitor. *Biochem Biophys Res Commun* **195:** 79–83, 1993.
13. Shimokado K, Yokota T, Umezawa K, Sasaguri T and Ogata J, Protein tyrosine kinase inhibitors inhibit chemotaxis of vascular smooth muscle cells. *Arterioscler Thromb* **14:** 973–981, 1994.
14. Abebe W and Agrawal DK, Role of tyrosine kinases in norepinephrine-induced contraction of vascular smooth muscle. *J Cardiovasc Pharmacol* **26:** 153–159, 1995.
15. Gould EM, Rembold CM and Murphy RA, Genistein, a tyrosine kinase inhibitor, reduces Ca^{2+} mobilization in swine carotid media. *Am J Physiol* **268:** C1425–C1429, 1995.
16. Honoré EK, Williams JK, Anthony MS and Clarkson TB, Soy isoflavones enhance coronary vascular reactivity in atherosclerotic female macaques. *Fertil Steril* **67:** 148–154, 1997.
17. Nestel PJ, Yamashita T, Sasahara T, Pomeroy S, Dart A, Komesaroff P, Owen A and Abbey M, Soy isoflavones improve systemic arterial compliance but not plasma lipids in menopausal and perimenopausal women. *Arterioscler Thromb Vasc Biol* **17:** 3392–3398, 1997.
18. Raines EW and Ross R, Biology of atherosclerotic plaque formation: Possible role of growth factors in lesion development and the potential impact of soy. *J Nutr* **125**: 624S–630S, 1995.
19. Fotsis T, Pepper M, Adlercreutz H, Hase T, Montesano R and Schweigerer L, Genistein, a dietary ingested isoflavonoid, inhibits cell proliferation and *in vitro* angiogenesis. *J Nutr* **125:** 790S–797S, 1995.
20. Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M and Fukami Y, Genistein, a specific

inhibitor of tyrosine-specific protein kinases. *J Biol Chem* **262:** 5592–5595, 1987.
21. Meng Q-H, Wähälä K, Adlercreutz H and Tikkanen MJ, Antiproliferative efficacy of lipophilic soy isoflavone phytoestrogens delivered by low density lipoprotein particles into cultured U937 cells. *Life Sci* **65:** 1695–1705, 1999.
22. Hochberg RB, Biological esterification of steroids. *Endocr Rev* **19:** 331–348, 1998.
23. Shwaery GT, Vita JA and Keaney JF Jr, Antioxidant protection of LDL by physiological concentrations of 17β-estradiol. Requirement for estradiol modification. *Circulation* **95:** 1378–1385, 1997.
24. Meng Q-H, Höckerstedt A, Heinonen S, Wähälä K, Adlercreutz H and Tikkanen MJ, Antioxidant protection of lipoproteins containing estrogens: *In vitro* evidence for low- and high-density lipoproteins as estrogen carriers. *Biochim Biophys Acta* **1439:** 331–340, 1999.
25. Tikkanen MJ, Wähälä K, Ojala S, Vihma V and Adlercreutz H, Effect of soybean phytoestrogen intake on low density lipoprotein oxidation resistance. *Proc Natl Acad Sci USA* **95:** 3106–3110, 1998.
26. Meng Q-H, Lewis P, Wähälä K, Adlercreutz H and Tikkanen MJ, Incorporation of esterified soybean isoflavones with antioxidant activity into low density lipoprotein. *Biochim Biophys Acta* **1438:** 369–376, 1999.
27. Janocko L and Hochberg RB, Estradiol fatty acid esters occur naturally in human blood. *Science* **22:** 1334–1336, 1983.
28. Hochberg RB, Pahuja SL, Zielinski JE and Larner JM, Steroidal fatty acid esters. *J Steroid Biochem Mol Biol* **40:** 577–585, 1991.
29. Larner JM, Pahuja SL, Shackleton CH, McMurray WJ, Giordano G and Hochberg RB, The isolation and characterization of estradiol-fatty acid esters in human ovarian follicular fluid. *J Biol Chem* **268:** 13893–13899, 1993.
30. Stampfer MJ and Colditz GA, Estrogen replacement therapy and coronary heart disease: A quantitative assessment of the epidemiologic evidence. *Prev Med* **20:** 47–63, 1991.
31. Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B and Vittinghoff E for the Heart and Estrogen/progestin Replacement Study (HERS) Research Group, Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. *JAMA* **280:** 605–613, 1998.
32. Kauffmann RF and Bryant HU, Selective estrogen receptor modulators. *Drug News Perspect* **8:** 531–539, 1995.
33. Hol T, Cox MB, Bryant HU and Draper MW, Selective estrogen receptor modulators and postmenopausal women's health. *J Women's Health* **6:** 523–531, 1997.
34. Cassidy A, Potential tissue selectivity of dietary phytoestrogens and estrogens. *Curr Opin Lipidol* **10:** 47–52, 1999.
35. Murkies AI, Wilcox G and Davis SR, Clinical review 92: Phytoestrogens. *J Clin Endocrinol Metab* **83:** 297–303, 1998.
36. Wang TTY, Sathyamoorthy N and Phang JM, Molecular effects of genistein on estrogen receptor mediated pathways. *Carcinogenesis* **17:** 271–275, 1996.
37. Adlercreutz H, Phytoestrogens: Epidemiology and possible role in cancer protection. *Environ Health Perspect* **103**(Suppl 7)**:** 103–112, 1995.
38. Mäkelä S, Savolainen H, Aavik E, Myllärniemi M, Strauss L, Taskinen E, Gustafsson J-Å and Häyry P, Differentiation between vasculoprotective and uterotrophic effects of ligands with different binding affinities to estrogen receptors α and β. *Proc Natl Acad Sci USA* **96:** 7077–7082, 1999.