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Isoflavones stimulate estrogen receptor-mediated core histone acetylation

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Abstract

The isoflavones genistein and daidzein and the daidzein metabolite equol have been reported to interact with estrogen receptors (ERs). Some studies indicate that they behave clinically like estrogen in some estrogen-deficiency diseases. However, the detailed molecular mechanism used by these compounds to create beneficial effects in patients with estrogen-related diseases has not been clarified. Using histone acetyltransferase (HAT) assay, we found that equol, genistein, and AglyMax had significant effects on ERα-mediated histone acetylation. Although 17β-estradiol (E2)-dependent HAT activity of steroid receptor coactivators 2 (SRC2) and p300 mediated by ERβ could be detected, it was weaker than that mediated by ERα. Equol, genistein, AglyMax, and daidzein all markedly stimulated ERβ-mediated histone acetylation. On the other hand, anti-estrogenic compounds ICI 182,780 (ICI) and tamoxifen (TA) did not have an effect on HAT activity mediated by either ERα or ERβ. Our data indicate that estrogenic ligands exert their effects by elevating histone acetylation and coactivator activity of ER, and suggest that the risk of estrogen-related diseases might be reduced by a sufficient amount of genistein or AglyMax supplements.

Keywords: Estrogen receptor; Histone acetylation; Estradiol; Genistein; Daidzein; Equol; AglyMax

The estrogen receptor (ER) is a ligand-dependent transcriptional factor that can bind with a number of ligands, including its natural hormone 17β-estradiol (E2) and environmental or synthetic chemicals. The transcriptional activities and functions of target tissues stimulated by these ligands are highly variable. Once bound by estrogens, the ER undergoes a conformational change influencing the cell growth, differentiation, and functions of many target tissues, such as the mammary gland, uterus, vagina, ovary, testes, and prostate [1]. It also plays an important role in bone metabolism and the functions of the cardiovascular and central nervous systems in both women and men [2–4]. Estrogen deficiency, especially in postmenopausal women, is related to various diseases including osteoporosis and arteriosclerosis.

Epidemiological study has suggested that consumption of soy based food helps to relieve postmenopausal symptoms, avoid cardiovascular disease, and maintain bone mass [5–8]. The soy isoflavones genistein and daidzein, which are the two principal components in soy products, are suggested to play an important role in these functions. Upon entering the human body, daidzein can be metabolized by bacteria in the large

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intestine to form the estrogenic equol and non-estrogenic *O*-desmethylangolensin, whereas genistein is metabolized to the non-estrogenic *P*-ethyl phenol [9,10]. Genistein, daidzein, and equol can all bind to ER because of their structural similarity to endogenous estrogens [11]. In clinical studies, equol has been shown to have strong estrogenic potency. For example, it has been reported that equol can prevent cardiovascular disease through inhibiting low-density lipoprotein oxidation and membrane lipid peroxidation [10,12], and has a positive effect on bone turnover [13]. However, the detailed molecular mechanism these components use to create these beneficial estrogenic effects on estrogen-related diseases has not been clarified.

Current studies have indicated that when a ligand such as estradiol binds, the ER exerts its action by binding to the estrogen response element (ERE) and stimulating the basal transcriptional machinery through interaction with a variety of coactivators [14]. In addition, increased core histone acetylation is thought to correlate with stimulated transcription through histone acetyltransferase (HAT) activity of the coactivators [15].

Recent studies have shown that p300 can bind directly to the ER A/B domain or indirectly to the ER through p160 proteins. Purified p300 functions cooperatively with estrogen-activated ER to enhance transcription by interacting with components of the basal transcriptional machinery such as RNA Pol II, TBP, and $TF_{II}B$, and facilitating an association with other transcription factors and coregulators [16,17]. Moreover, P300 has been found to possess histone acetyltransferase activity that acetylates both histone and non-histone proteins, such as ER and the p160 family [18].

Another factor, SRC2, belongs to the p160 family, which includes steroid receptor coactivator 1 (SRC1); glucocorticoid receptor interaction protein 1 (GRIP1)/transcriptional intermediary protein 2 (TIF2)/SRC2; and activator of thyroid and retinoic acid receptors (ACTRs)/amplified in breast cancer 1 (AIB1)/receptor-associated coactivator 3 (RAC3)/thyroid receptor activator molecule 1(TRAM1)/pCIP/SRC3 [19]. The p160 coactivators function as bridging factors to recruit p300/CBP to chromatin-bound ERα. They interact with the activation function-2 (AF2) domain of nuclear receptors through their LXXLL signature motifs in a ligand-dependent manner [20]. Kim et al. [21] have shown that SRC2 is necessary and sufficient for the recruitment of p300 HAT activity to chromatin-bound ERα. It was found that E2 stimulated HAT activity of p300 in the presence of ERα and SRC2. In the current study, we examined the ability of various ligands such as genistein, daidzein, equol, and the synthetic chemical AglyMax to stimulate the HAT activity of p300. Two anti-estrogenic compounds ICI and tamoxifen (TA) were also examined. In this study, we also tested these ligands using ERβ in addition to ERα.

Materials and methods

Chemicals. 17β-Estradiol (>98% pure) and tamoxifen (>99% pure) were obtained from Sigma (Tokyo, Japan), whereas genistein (>98% pure) and daidzein (>98% pure) were from Wako (Osaka, Japan). AglyMax-70 was from Nichimo (Tokyo, Japan) and equol was obtained from Extrasynthese (Genay, France). ICI 182,780 was kindly provided by AstraZeneca (London, UK).

Synthesis and purification of recombinant proteins. His-tagged p300 and flag-tagged ERα and β were synthesized in sf9 cells using a baculovirus expression system. Three days after infection, purification was performed using Ni–NTA agarose for p300, and flag M2-Agarose for both ERα and β. For GST-tagged SRC2 purification, BL21 cells were transformed with plasmid for expression of the SRC2 construct. Cells were grown at 37 °C until the OD_{600} reached 0.6. After that, SRC2 expression was induced with 1 mM IPTG at 30 °C for 4 h. SRC2 was purified with Glutathione Sepharose 4 Fast Flow (Amersham Biosciences, Sweden). The purified proteins were subjected to 11% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and then visualized by Coomassie brilliant blue R staining.

Histone acetylation assay. Chromatin was made by salt dialysis as previously described [22]. Chromatin was made by mixing the pERE plasmid template and *Drosophila* core histone octomer in the presence of high salt, with subsequent salt dialysis and purification using a linear 15–40% (w/v) glycerol gradient. For the acetylation assay, the incubation temperature of the whole interaction was performed at 27 °C. At first, 1 μl aliquot of chromatin containing 62.5 ng of plasmid DNA and 62.5 ng of histones was mixed with 3 mM ATP and 2 mM $MgCl_2$ for an hour. Then, various combinations of proteins [p300 (0.02 pM), SRC2 (0.02 pM), ERα (0.14 pM), and ERβ (0.14 pM)] were added as indicated. After 20 min, 6.67 mM sodium butyrate, 7.4 kBq [^{3}H]acetylCoA, and 50 mM KCl–HEG were added in a final volume of 7.5 μl. Finally, the reaction was incubated at 27 °C for 2.5 h and analyzed on 13.5% SDS–PAGE with subsequent fluorography.

Results

Isoflavones stimulated core histone acetylation mediated by ERα

To examine the role of various ligands in ERα-mediated HAT activity, we expressed and purified ERα and β, p300, and SRC2 as shown in Fig. 1. First, we tested the role of ERα, E2, and coactivators p300 or SRC2 in core histone acetylation. As shown in Fig. 2A, p300 HAT was essential for histone acetylation (compare lanes 1 and 10 with the others), consistent with previous findings. Significant HAT activity was detected only in the presence of ERα, SRC2, and p300 together with E2. All of the factors are essential for maximum HAT activity. These results were consistent with previous observation by Kraus's group [21].

Based on the results shown in Fig. 2A, we then tested the HAT activities induced by various estrogenic ligands, including E2, AglyMax, genistein, equol, and daidzein. Different concentrations of these ligands were used for each lane, varying from 12.5 nM to 12.8 μM. We found that equol, genistein, and AglyMax had significant effects on histone acetylation mediated by ERα, and HAT activities were stimulated by these ligands in a

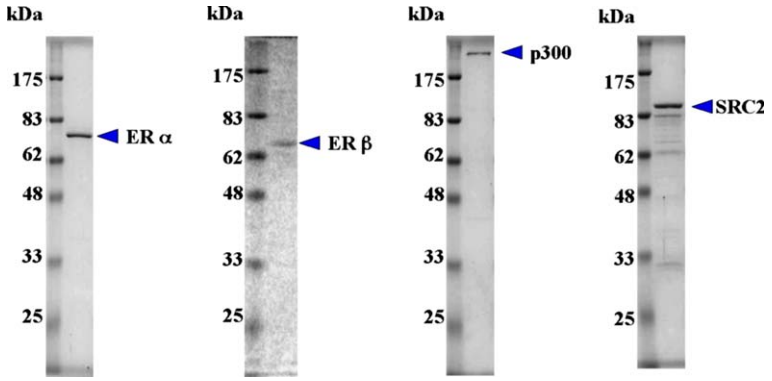

Fig. 1. Expression and purification of recombinant His-tagged p300, GST-tagged SRC2, and Flag-tagged ERα and β. His-tagged p300 and Flag-tagged ERα and β were expressed in sf9 cells using a baculovirus expression system. GST-tagged SRC2 was synthesized in BL21 cells. After purification, each protein was subjected to 11% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and then visualized by Coomassie brilliant blue R staining.

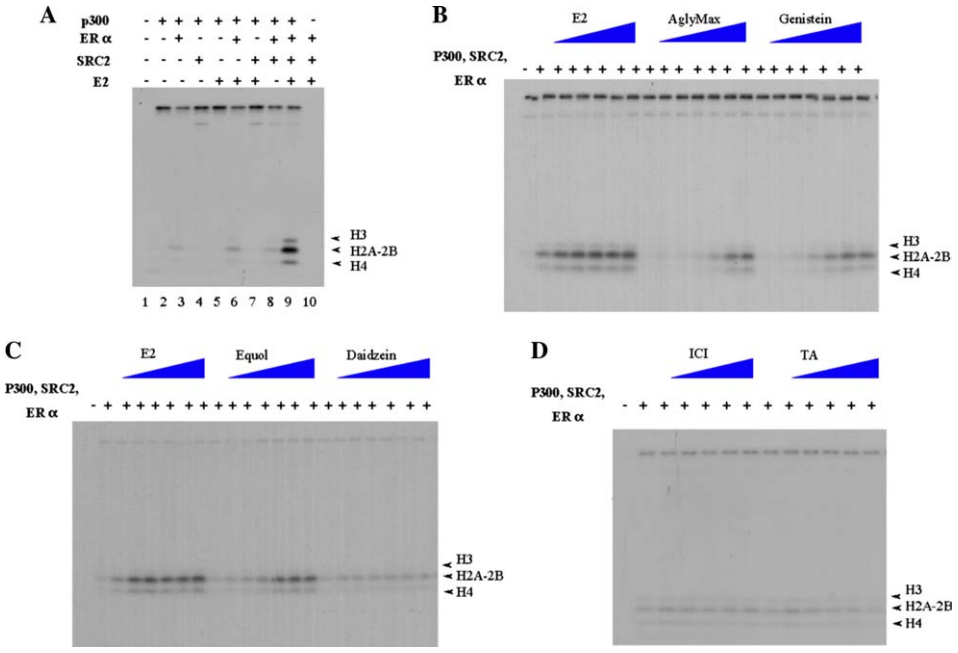

Fig. 2. Isoflavones stimulate core histone acetylation in the presence of ERα and its coactivators p300 and SRC2. (A) ERα-mediated ligand-dependent histone acetylation required coactivators of p300 and SRC2. In addition to ERα and E2, a reaction was performed with chromatin, [^{3}H]acetylCoA, and coactivators as indicated. After incubation, the reactions were subjected to electrophoresis on 13.5% SDS–PAGE with subsequent fluorography. Core histones (H2A, H2B, H3, and H4) are indicated with arrows. (B) E2, AglyMax, and genistein stimulated ERα-mediated core histone acetylation significantly. E2, AglyMax, genistein, ERα, P300, and SRC2 were added to reactions as indicated. The concentrations of ligands were examined from 12.5 nM to 12.8 μM. (C) E2, equol, and daidzein stimulated ERα-mediated core histone acetylation. (D) ICI and TA did not have an effect on ERα-mediated core histone acetylation. The concentrations of ligands were tested from 50 nM to 12.8 μM.

dose-dependent manner. The order of effect as ligands to HAT activity was: E2 > equol > genistein > AglyMax > daidzein (Figs. 2B and C). We also observed that ICI and TA did not have an effect under the same conditions (Fig. 2D).

Isoflavones stimulated core histone acetylation mediated by ERβ

Although ERβ–E2-dependent HAT activity was about 2.1-fold weaker than that mediated by ERα and E2 under the same conditions (Fig. 3), ligand dependency of ERβ is similar to that of ERα. As shown in Fig. 4A, significant HAT activity was detected only in the presence of ERβ, SRC2, and p300 together with E2. All of the factors are essential for maximum HAT activity.

We also examined the potency of various estrogenic ligands in stimulating ERβ-mediated core histone acetylation. All of these ligands stimulated ERβ-mediated HAT activity markedly (Figs. 4B and C). Both anti-estrogens ICI and TA had no effect on ERβ-mediated core histone acetylation (Fig. 4D).

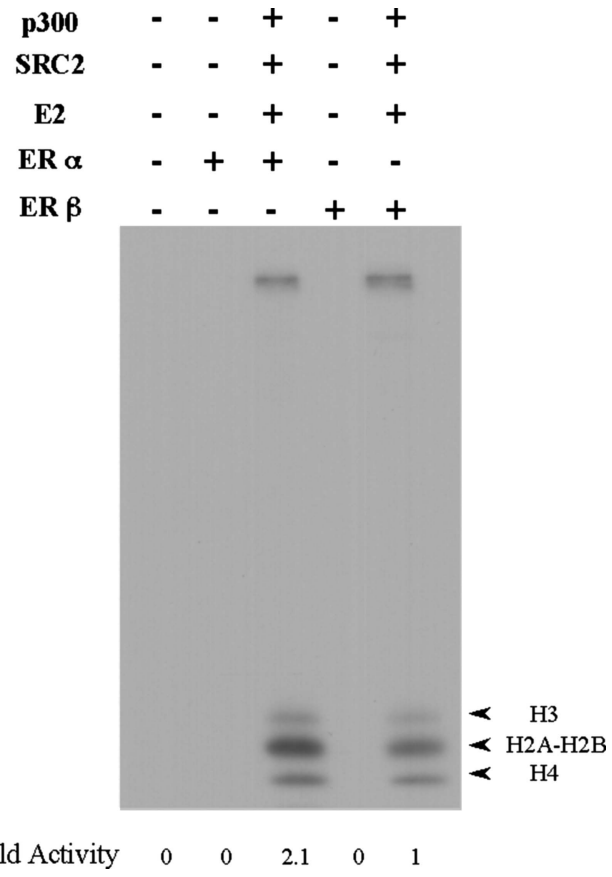

Fig. 3. Comparison of E2-dependent HAT activity mediated by ERα with that mediated by ERβ. The histone acetylation assay was carried out using chromatin with ERE. ERα and β were added together with coactivators as indicated.

Discussion

In this study, we found that genistein, equol, and AglyMax have significant effects on histone acetylation-mediated ERα. AglyMax is a synthetic chemical, containing 49.8% daidzein, 14.9% glycitein, and 6% genistein. According to the measurement of signals using densitometry, the potency of genistein is about 256-fold less than estradiol, and AglyMax is a little weaker than genistein. This suggests that we might be able to reduce the risk of estrogen-related diseases with sufficient amounts of genistein or AglyMax supplements, and thus it is desirable to discuss serum concentration of these hormones.

A woman's serum concentration of estradiol ranges from 80.76 pM to 1.73 nM, varying with age, estrous cycle, tissues, and other factors [23]. Because of the sudden estradiol decrease postmenopause, women may suffer from related symptoms such as osteoporosis and arteriosclerosis [24–26]. Serum concentrations of genistein, daidzein, and equol are quite different among women in various geographic areas because of major differences in soy consumption. Some studies have examined the serum concentrations of these phytoestrogens in women who are over 40 years old. It has been reported that in the United Kingdom, serum concentration is about 23–33 nM for genistein, 10–15 nM for daidzein, and 1.2–3.2 nM for equol [27]. These serum concentration levels are significantly lower than those in Japanese women, who are thought to be one of the highest consumers of soy and soy products. The serum concentration in Japanese women is from 376 to 627 nM for genistein, 182 to 312 nM for daidzein, and 35 to 80 nM for equol. So we tested the concentration of phytoestrogens in the range from 12.5 nM to 12.8 μM in our assay. We also found that HAT activity stimulated by 200 nM E2 could be inhibited by ICI or TA at a concentration $\geqslant$800 nM (data not shown). Thus, these in vitro data provide important information for hormone replacement therapy using phytoestrogens and other drugs such as ICI and TA. In addition, it has also been reported that after treatment with phytoestrogens, serum concentration could be increased 20-fold for genistein, 106-fold for daidzein, and 20-fold for equol [28]. Other studies have also reported that full isoflavone intake could elevate serum concentration even more, up to 1 μM [1]. Based on relative HAT activity, 12.8 μM genistein has about the same effect as 50 nM estradiol; thus a 500 nM serum genistein concentration might have the same effect as 2 nM estradiol. Therefore, at this level, genistein, in addition to intrinsic estradiol, might influence the ERα significantly. AglyMax has an almost equivalent effect also. The beneficial effect of isoflavones might be explained by ERα, its coactivators, and ligand-dependent core histone acetylation.

In addition to ERα, we also tested the effect of these compounds on ERβ. Although the effects of p300 and SRC2 on ERβ-mediated HAT are similar to that on ERα, its activity was about 2.1-fold weaker than ERα under the same reaction conditions. This result is consistent with previous studies. Kuiper et al. [1] found that E2-stimulated reporter gene activity by ERβ is lower than that obtained by ERα in human 293 embryonal kidney cells. Monroe et al. [29] indicated that SRC2 consistently enhances ERα-dependent transcription to a greater extent than ERβ in both human fetal osteoblast or monkey kidney cell lines.

The expressions of ERα and β are different in various tissues. ERα is predominantly expressed in tissues such as pituitary, uterus, and mammary gland, whereas ERβ is expressed in tissues such as brain, bone, and bladder [5,30]. In vitro, the ability of phytoestrogens to stimulate HAT activity is slightly different for ERα and β. However, in vivo, because of the tissue-specific expression of ER, phytoestrogens might have a role through both ERα and β, depending on the tissue.

The results of this study are consistent with the model that ER coactivator P300 acetylates core histone in the presence of SRC2 when isoflavone binds to ER (Fig. 5).

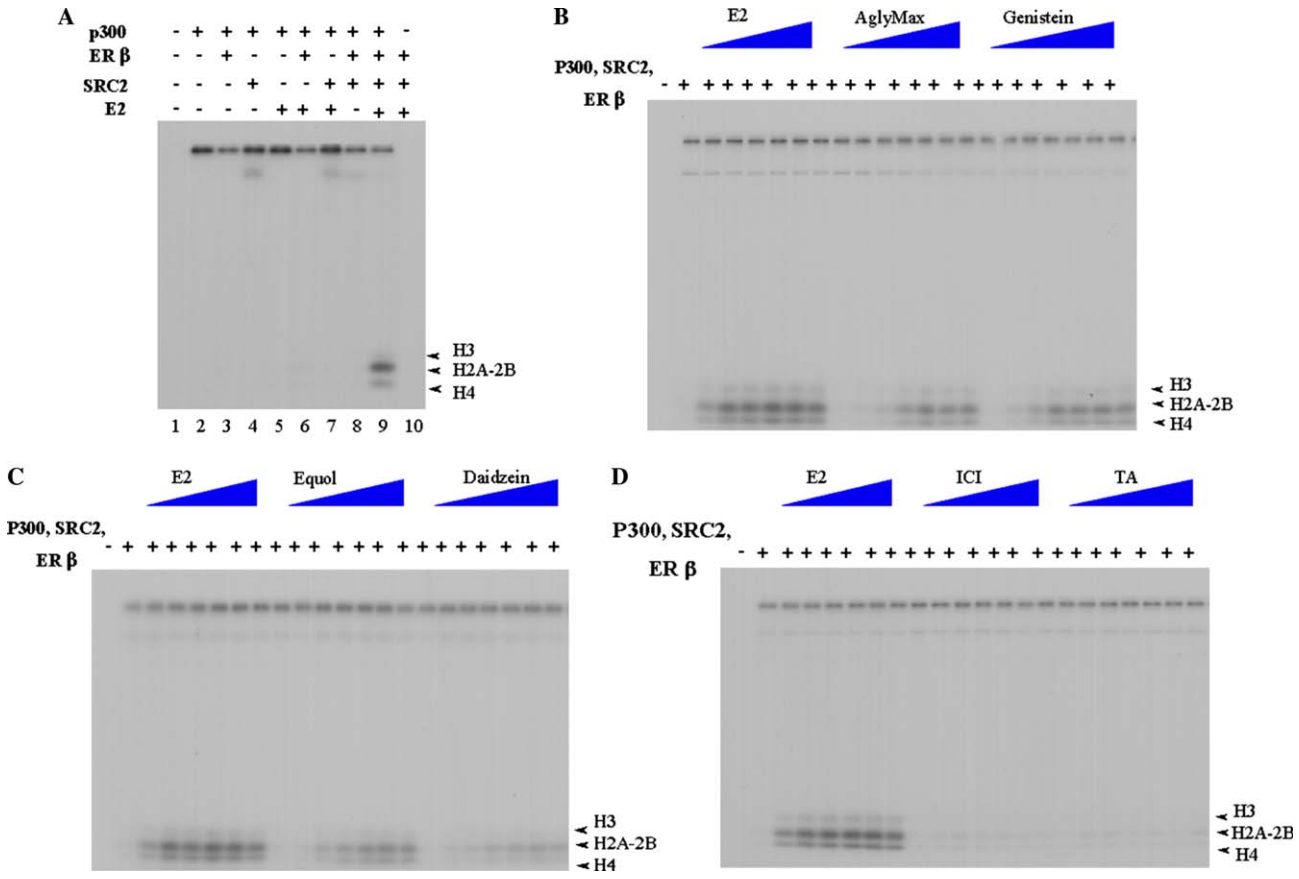

Fig. 4. Isoflavones stimulate core histone acetylation in the presence of ERβ and its coactivators p300 and SRC2. (A) ERβ-mediated ligand-dependent histone acetylation required p300 and SRC2 coactivators. In addition to ERβ and E2, reaction was performed with chromatin, [^{3}H]acetylCoA, and coactivators as indicated. After incubation, the reactions were subjected to 13.5% SDS–PAGE with subsequent fluorography. Core histones (H2A, H2B, H3, and H4) are indicated with arrows. (B) E2, AglyMax, and genistein stimulated ERβ-mediated core histone acetylation significantly. E2, AglyMax, genistein, ERβ, p300, and SRC2 were added to reactions as indicated. (C) E2, equol, and daidzein stimulated ERβ-mediated core histone acetylation. (D) ICI and TA did not affect ERβ-mediated core histone acetylation. The concentrations of ligands were examined from 12.5 nM to 12.8 μM.

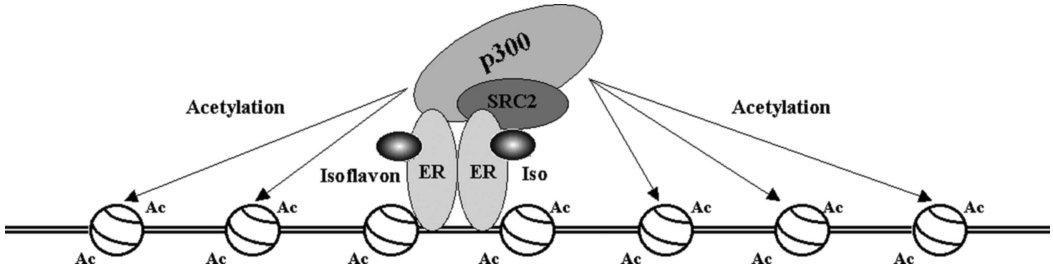

Fig. 5. Model for transcriptional activation by ER. ER and its coactivators acetylate core histones as well as its native ligand in an isoflavone-dependent manner. Iso, isoflavone.

In conclusion, this study indicates that estrogenic chemicals such as equol, genistein, and AglyMax have significant effects on ER-mediated core histone acetylation, and daidzein also has a strong ability to stimulate ERβ-mediated core histone acetylation. All of these estrogenic ligands exert their effects by elevating histone acetylation and coactivator activity of ER. This suggests that we might be able to reduce the risk of estrogen-related diseases with sufficient amounts of genistein or AglyMax supplements.

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