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Journal of Steroid Biochemistry & Molecular Biology 91 (2004) 241–246
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Estrogenic activity of glabridin and glabrene from licorice roots on human osteoblasts and prepubertal rat skeletal tissues

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Received 13 November 2003; accepted 29 April 2004

Abstract

Data from both in vivo and in vitro experiments demonstrated that glabridin and glabrene are similar to estradiol-17β in their stimulation of the specific activity of creatine kinase, although at higher concentrations, but differ in their extent of action and interaction with other drugs. In pre-menopausal human bone cells, the response to estradiol-17β and glabridin (at higher concentration) was higher than in post-menopausal cells; whereas, glabrene (at higher concentration) was more effective in post-menopausal cells. At both ages, the response to estradiol-17β and glabridin was enhanced by pretreatment with the less-calcemic Vitamin D analog CB 1093 (CB) and the demonstrably non-calcemic analog JK 1624 F_2-2 (JKF). The response to glabrene was reduced by this pretreatment. Both glabridin and glabrene stimulated creatine kinase specific activity in diaphyseal bone and epiphyseal cartilage of prepubertal female rats. Daily feeding (3–14 days) of prepubertal female rats with glabridin, estradiol-17β or their combination, also stimulated creatine kinase specific activity. Glabridine, similarly to estradiol-17β, also stimulated creatine kinase specific activity in ovariectomized female rats. Raloxifene, in combination with glabridin or estradiol-17β, demonstrated the phenomenon of mutual annihilation of stimulation of creatine kinase specific activity in both epiphysis and diaphysis. Glabrene activity was not inhibited by raloxifene. Therefore, glabridin shows greater similarity to estradiol-17β and thus greater potential, with or without Vitamin D, to modulate bone disorders in post-menopausal women.

Keywords: Glabridin; Glabrene; Estradiol; Vitamin D analogs raloxifene; Creatine kinase; Human bone cells; Epiphysis; Diaphysis

1. Introduction

The constituents of licorice, originally isolated from aqueous extracts include glycyrrhizin and its aglycone, glycyrrhetinic acid, which are used in the treatment of hyperlipemia, atherosclerosis, viral diseases and allergic inflammation [1]. The acetone or ethanol extract of licorice root contains sub-classes of the flavonoid family, isoflavans, isoflavenes and chalcones, such as glabridin, glabrol, glabrene, 3-hydroxyglabrol, 4′-*O*-methylglabridin hispaglabridin A and hispaglabridin B [2–4]. Licorice root is indeed one of the richest sources of the isoflavans and glabridin, present in the extract at more than 10% (w/w), is the major compound in this class. It exhibits diverse biological activities [5–8], including estrogen-like activity [5–7], which was initially suggested by the similarity of the structure and lipophilicity of glabridin to that of estradiol. Studies in vivo suggested that there might be more compounds in the extract, which contribute to its estrogen-like activity. This led

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doi:10.1016/j.jsbmb.2004.04.008

us to identify other active constituents, such as glabrene, an isoflavene, which binds to the human estrogen receptor with about the same affinity as glabridin or genistein [6,7].

Estrogen deficiency is known to be involved in osteoporosis [9,10], which affects every third woman above the age of 65. It has been recently reported that estrogens as well as androgens are also necessary for the maintenance of the male skeleton (reviewed in [11]). Osteoporosis is characterized by a reduction in bone density and strength to the extent that fractures occur after minimal trauma. At menopause, an accelerated loss of bone mass (3%/year) takes place during the first 5 years, along with changes in bone structure. It has been suggested that in estradiol deficiency, the loss of regulation of the lifespan of osteoclasts may be mainly responsible for the imbalance between formation and resorption and the resulting progressive loss of bone mass and strength [10]. Estrogen stimulates osteoblast formation, decreases the number of osteoblasts, slows the rate of bone remodeling and thus protects against bone loss. Although estrogen-treatment is efficient in preventing bone loss, it can also contribute to the development of estrogen-dependent tumors such as endometrial and breast tumors. Hence, new compounds, which can replace current hormone replacement therapy treatments with no such deleterious effects, are highly desirable.

In the present study, the effects of glabridin and glabrene on the skeletal system were tested in vitro in cultured human female bone cells and in vivo in female rat skeletal tissues. The stimulation of creatine kinase specific activity (CK) in epiphysis and diaphysis of the bone [12] as well as in cultured human female bone cells at different ages was measured. CK activity is a convenient estrogen response marker, since it is induced by estrogens in vivo and in vitro [13,14].

2. Materials and methods

2.1. Reagents

All reagents were of analytical grade. Chemicals were purchased from Sigma (St. Louis, MO). Glabridin and glabrene were isolated from licorice roots [2]; CB 1093 was a gift of Leo Pharmaceutical products Ballerup Denmark. JK 1624 F_2-2 was synthesized as previously described [15]. Raloxifene was extracted from (Evista®) tablets.

2.2. Cell cultures

Human female bone cells from pre- and post-menopausal women were prepared from bone explants, by a non-enzymic method as described previously [16]. Samples of the trabecular surface of the iliac crest or long bones were cut into 1 mm^3 pieces and repeatedly washed with phosphate-buffered saline to remove blood components. The explants were incubated in DMEM medium without calcium (to avoid fibroblastic growth) containing 10% fetal calf serum (FCS) and antibiotics. First passage cells were seeded at a density of 3×10^5 cells/35 mm tissue culture dish, in phenol red-free DMEM with 10% charcoal stripped FCS, and incubated at 37 °C in 5% CO_2.

2.3. Creatine kinase extraction and assay in human osteoblasts

Cells were treated for 24 h with the various agents as specified, scraped off and homogenized by freezing and thawing three times in an extraction buffer, as previously described [17–20]. Supernatant extracts were obtained by centrifugation of homogenates at 14,000 × *g* for 5 min at 4 °C in an Eppendorf micro-centrifuge. Creatine kinase activity was assayed by a coupled spectrophotometric assay, as previously described [17–20]. Protein was determined by Coomasie blue dye binding, using bovine serum albumin (BSA) as the standard.

2.4. Animals

Immature female Wistar rats (25 days old) were used intact, or 2 weeks post-ovariectomy. The rats were housed in air-conditioned quarters with light from 05:00 to 19:00 h, and food and water ad libitum. All experiments were carried out according to the regulations of the Committee on Experimental Animals of the Tel-Aviv Sourasky Medical Center.

2.5. Creatine kinase extraction and assay in rat organs

Changes in the specific activity of CK in epiphyseal cartilage and in diaphyseal bone, induced by a single intraperitoneal injection (i.p.) of estradiol-17β (E_2), glabridin or glabrene, were assayed in 25-day-old immature female rats or in ovariectomized female rats, 2 weeks after surgery. Matched control rats were injected with vehicle: 0.05% ethanol in phosphate-buffered saline. E_2 was injected at 5 μg for immature and 10 μg for ovariectomized female rats [20], and rats were sacrificed 24 h after the injection. For multiple applications, rats were fed directly by stomach tube daily for 4 days or injected for 2 weeks (5 days per week) at the indicated doses. The organs to be examined were removed and stored at −20 °C until processed for assay of CK, as described [16,20].

2.6. Statistical analysis

Differences between the mean values of experimental and control groups were evaluated by analysis of variance (ANOVA). *P*-values less than 0.05 were considered significant.

3. Results

3.1. Different effects of glabridin and glabrene combined with non-hypercalcemic Vitamin D analogs, on CK specific activity in human female bone cells

To determine their responsiveness to glabridin and glabrene, human bone cells, were incubated with 30 nM E_2,

or 3000 nM glabridin or glabrene. An increase in CK specific activity was found in both pre- and post-menopausal cells (Fig. 1), similar to that caused by E_2, although at 100-fold higher concentrations. However, the increase was greater in the case of glabridin in pre-menopausal cells but after glabrene in post-menopausal cells.

Moreover, when human female bone cells were pre-incubated with a less-calcemic Vitamin D analog, CB 1093 (1 nM,) by daily treatment for 3 days and then treated for 24 h with either E_2 (30 nM), glabridin at 100-fold higher concentration (3000 nM) or glabrene at 100-fold higher concentration (3000 nM), there was a significant enhancement of the stimulation of CK synthesis by E_2 or glabridin; the response to glabrene was completely inhibited in pre-menopausal cells and decreased in post-menopausal cells (Fig. 2). The demonstrably non-calcemic Vitamin D analog JK 1624 F_2-2 (JKF) similarly enhanced the stimulation of CK activity by E_2, or glabridin and reduced the response to glabrene in both pre- and post-menopausal derived cells (Fig. 2).

3.2. Increase of CK specific activity in skeletal tissues by a single injection of glabridin or glabrene into immature female rats and its inhibition by raloxifene

Immature female rats, weighing about 60 g, were injected with increasing concentrations of glabridin or glabrene, ranging from 3 to 300 μg/rat.(Fig. 3) Stimulation of CK by glabridin or glabrene in diaphyseal bone (Di) and epiphyseal cartilage (Ep) showed a significant increase relative to vehicle-treated controls at 3 μg, the lowest dose tested, and reached a near plateau between 25 and 300 μg. In both organs, the response to glabridin was significantly higher than to glabrene (Fig. 3).

0.0 When immature female rats were injected with glabridin or glabrene at the maximal dosage (300 μg) or with E_2 (5 μg), together with 500 μg raloxifene, the stimulation of CK specific activity in Di and Ep by glabridin, or E_2, but not that of glabrene, was completely blocked (Fig. 4).

3.3. Repeated oral administration of glabridin, or estradiol, in immature female rats increases CK specific activity

Daily feeding of glabridin (at concentrations of 2.5–250 μg/rat/day) or E_2 (0.5–5 μg/rat/day), for 3 days, stimulated CK specific activity in Di to a greater extent than in Ep (Fig. 5). When the daily feeding with 2.5 μg/rat/day E_2 or 25 μg/rat/day of glabridin or both, was extended for 2

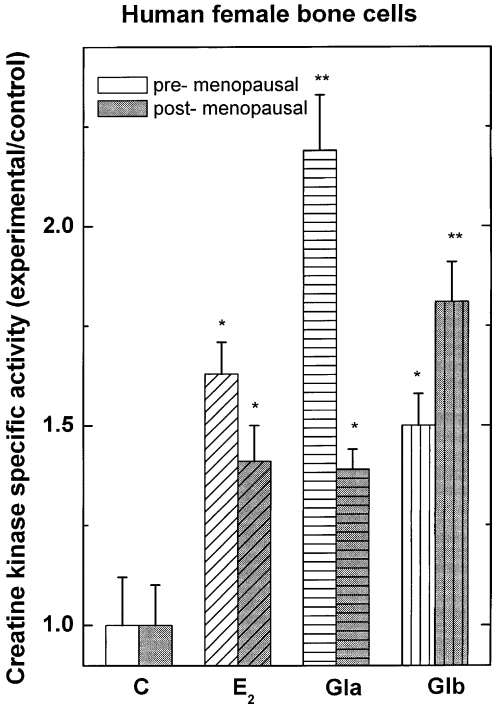

Fig. 1. Stimulation by E_2 (30 nM), glabridin (Gla) or glabrene (Glb) (3000 nM) of creatine kinase (CK) specific activity in human female bone cells. Results are mean ± S.E.M. of triplicate cultures from five pre-menopausal and seven post-menopausal women expressed as the ratio between CK specific activity in hormone-treated and control cells. $^{*}P < 0.05$, $^{**}P < 0.01$. Basal activity of creatine kinase: 0.020 ± 0.005 μmol/min/mg (pre-menopausal), 0.016 ± 0.004 μmol/min/mg protein (post-menopausal).

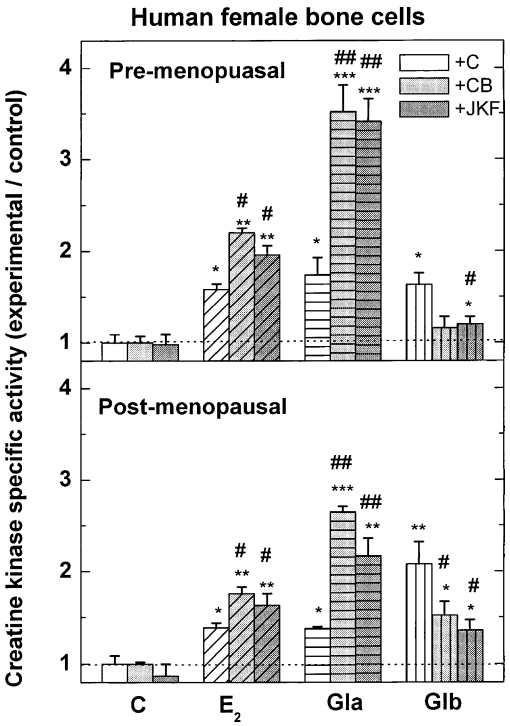

Fig. 2. The effect of pre-treatment with Vitamin D analogs CB 1089 (CB) or JK 1624 F_2-2 (JKF), at 1 nM by daily additions for 3 days, on the stimulation by E_2 (30 nM), glabridin (Gla) (3000 nM) or glabrene (Glb (3000 nM), of CK specific activity in human female bone cells. Results are mean ± S.E.M. of triplicate cultures from three women at each age group, expressed as the ratio between CK specific activity in hormone-treated and control cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Comparison of activity of estrogen-treatment after Vitamin D pretreatment vs. estrogen-treatment after vehicle pretreatment $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. The basal activity of creatine kinase was as reported in Fig. 1.

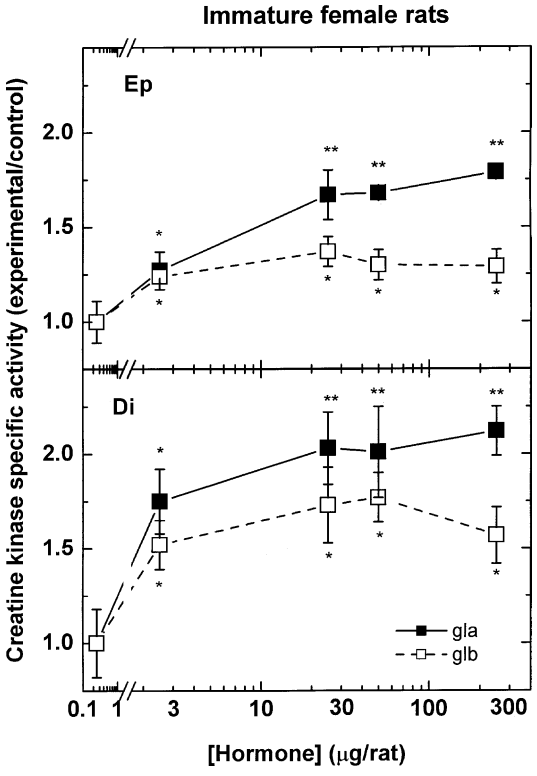

Fig. 3. Dose-dependent stimulation, by a single injection of glabridin (gla) or glabrene (glb), of creatine kinase specific activity in epiphyseal cartilage (Ep) and in diaphyseal bone (Di) of immature female rats. Results are mean ± S.E.M. (n = 4) expressed as the ratio between the specific activity in hormone-treated and control tissues. $^{*}P < 0.05$, $^{**}P < 0.01$. The basal activity of creatine kinase was 1.13 ± 0.16 μmol/min/mg protein (Ep) and 1.30 ± 0.03 μmol/min/mg protein (Di).

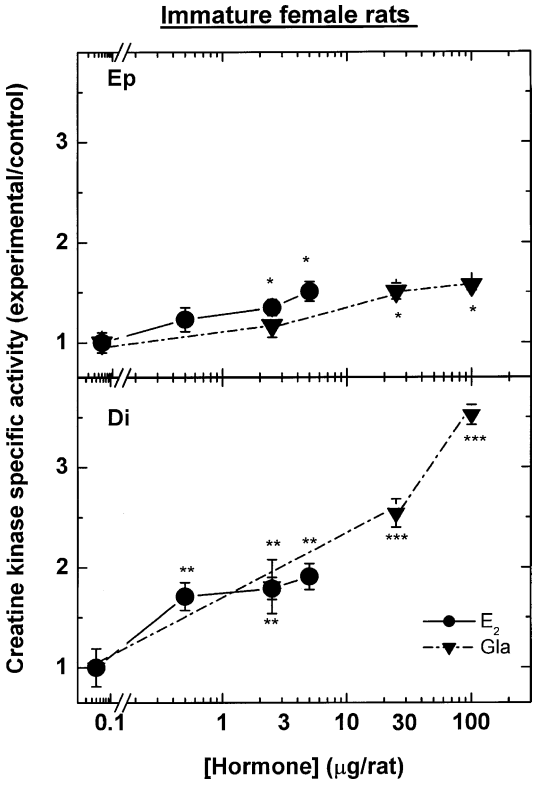

Fig. 5. Dose-dependent stimulation by three daily feedings of glabridin (Gla, 2.5–100 μg/rat/day) or E_2 (0.5–2.5 μg/rat/day) of creatine kinase specific activity in epiphyseal cartilage (Ep) and in diaphyseal bone (Di) of immature female rats. Results are mean ± S.E.M. (n = 6) expressed as the ratio between the specific activity hormone-treated and control tissues. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The basal activity of CK was 1.53 ± 0.26 μmol/min/mg protein (Ep) and 1.20 ± 0.13 μmol/min/mg protein (Di).

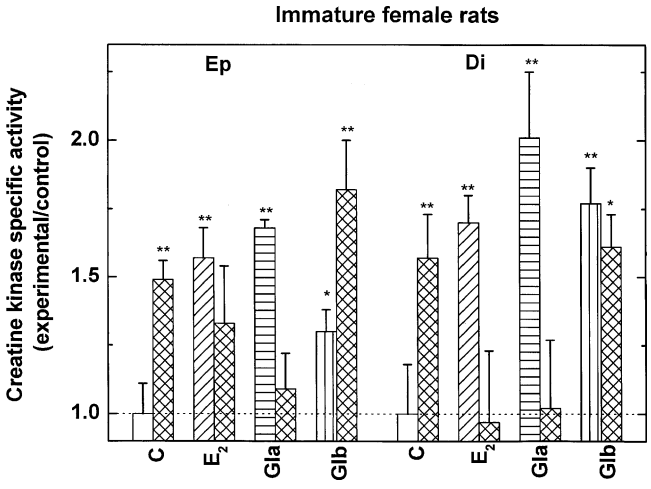

Fig. 4. The effect of glabridin (Gla, 50 μg), glabrene (Glb, 50 μg) or E_2 (5 μg), alone or in the presence of raloxifene (500 μg, cross hatched bars) on creatine kinase specific activity in epiphyseal cartilage (Ep) and diaphyseal bone (Di) of immature female rats. Results are mean ± S.E.M. (n = 4) expressed as the ratio between the specific activity in hormone-treated and control tissues. $^{*}P < 0.05$, $^{**}P < 0.01$. The basal activity of CK was similar to that shown in Fig. 3.

weeks, the stimulation by glabridin, although at higher concentrations, was similar to the effect of E_2 in both organs, and the stimulation by the combined treatment was significantly greater than the effect of either E_2 or glabridin alone (Fig. 6).

3.4. A single injection of glabridin or estradiol in ovariectomized female rats increases CK specific activity

To make the in vivo model a closer parallel to the human female bone cells studied above (Figs. 1 and 2), ovariectomized female rats were injected with glabridin at the maximal concentration used for adult rats (100 μg/rat) or with E_2 (10 μg/rat). The specific activities of CK in Di and in Ep were stimulated (highly significantly) to a similar extent by glabridin or E_2 although at higher concentrations (Fig. 7).

4. Discussion

While both glabridin and glabrene showed estrogenic activity, in vivo and in vitro, we found a clear distinction in their pattern of action. In human female bone cells, glabridin, similarly to E_2, increased CK specific activity more

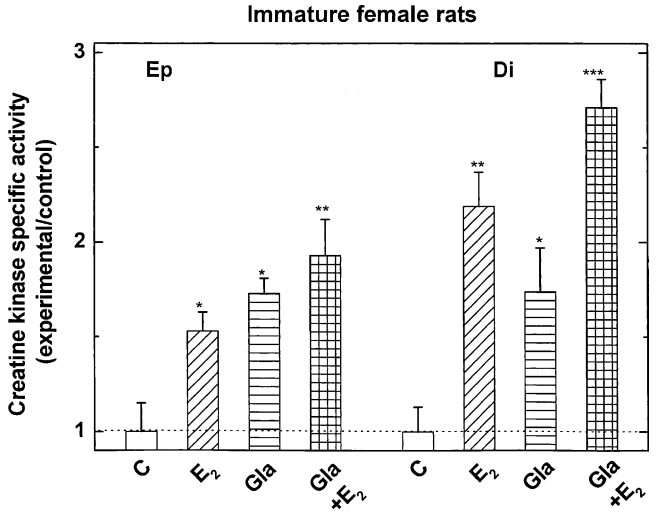

Fig. 6. The effect of 2 weeks' daily feeding (5 days/week) of glabridin (Gla, 25 μg/rat/day) or E_2 (2.5 μg/rat/day) or both, on creatine kinase specific activity in epiphyseal cartilage (Ep) and in diaphyseal bone (Di) of immature female rats. Results are mean ± S.E.M. (n = 10) expressed as the ratio between the specific activity in hormone-treated and control tissues. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The basal activity of creatine kinase was 1.53 ± 0.2609 μmol/ min/mg protein (Ep) and 1.15 ± 0.09 μmol/min/mg protein (Di).

in pre-menopausal cells, while glabrene had a greater effect in post-menopausal cells. In prepubertal female rats, glabridin showed a greater stimulation of CK specific activity in both diaphyseal bone and epiphyseal cartilage than glabrene. Moreover, in the presence of the non-hyper-calcemic Vitamin D analogs CB [14] or JKF [15], glabridin and E_2 enhanced CK specific activity, while the effect of glabrene was inhibited. In the presence of raloxifene, an antagonist of E_2, glabridin's

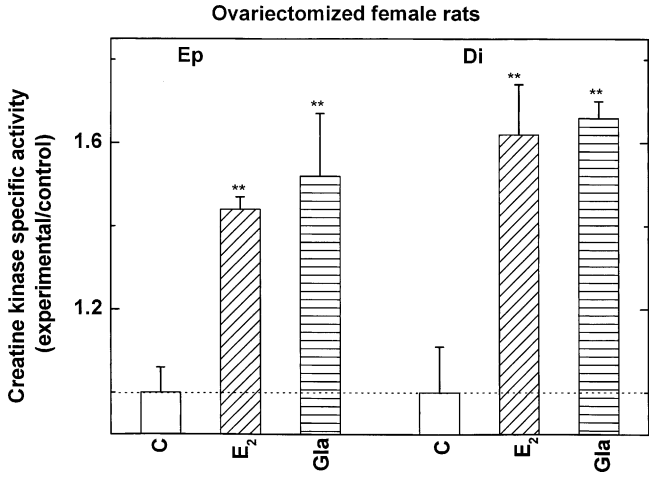

Fig. 7. The effect of single injection of glabridin (Gla 100 μg) or E_2 (10 μg) on creatine kinase specific activity in epiphyseal cartilage (Ep) and in diaphyseal bone (Di) of ovariectomized female rats. Results are mean ± S.E.M. (n = 10) expressed as the ratio between the specific activities in hormone-treated and control tissues. $^{*}P < 0.05$, $^{**}P < 0.01$. The basal activity of creatine kinase was 0.49 ± 0.27 μmol/min/mg protein (Ep) and 1.26 ± 0.22 μmol/min/mg protein (Di).

stimulation was inhibited to the same extent as that of E_2, but glabrene's stimulation was not affected, which suggests the existence of an ER independent mechanism for glabrene. Such a distinction between a phytoestrogen and estradiol is shown for example, by the structurally related isoflavone, ipriflavone, which prevents ovariectomy-associated bone loss by a different mechanism than E_2 [21].

In previous studies, in rat models, we reported that Vitamin D analogs enhance response and sensitivity to E_2 in ROS 17/2.8 osteoblast-like cells [14], rat embryo cells [22] and bone and cartilage in vivo [20]. These analogs increased the sensitivity to E_2 by lowering the dose for a comparable response to E_2 by one or two orders of magnitude. In the present study, the Vitamin D analogs JKF [15] and CB [14], which by themselves did not stimulate CK specific activity, significantly increased the CK response to glabridin. These results suggest that combined treatment with non-hypercalcemic Vitamin D analogs, by increasing the effects of glabridin, may permit the use of lower doses of glabridin in any clinical application.

The necessary in vivo dose–response study of glabridin and glabrene performed in immature rats revealed significant increases at a dose as low as 3 μg/rat, a low dose for a phytoestrogen. The plateau dose of 50 μg/rat stimulated CK specific activity to a slightly greater extent in diaphyseal bones than in epiphyseal cartilage. This dose of glabridin was used to test the effects of raloxifene, which was found to inhibit CK stimulation by glabridin, or E_2 as it had inhibited their effects on cardiovascular tissues [23], suggesting a mutual ER(s)-dependent mechanism. Additionally, glabridin like raloxifene behaves like a SERM (Fig. 4), exhibiting the phenomenon of mutual annihilation [24], namely, inhibiting each other activity as well as E_2 activity, which has been suggested to result from the formation of mixed heterodimers of ligand–ER(s) monomers. The high degree of conformational specificity of this interaction is shown in the fact that several other phytoestrogens including genistein and resveratrol [24] do not exhibit this phenomenon.

In the present study, when immature rats were fed glabridin or E_2, for 3 days, CK activity was also found to be higher in Di than in Ep, but after 2 weeks, the effects were comparable, which suggests tissue specific differences in short and longer term responses in cartilage and bone.

The stimulation of CK activity by glabridin in the more clinically relevant model of ovariectomized female rats verified the above effects on immature rats suggesting a possible role in therapeutic regulation of bone growth similar to the estrogenic effects of genistein on bone metabolism found in vitro and in vivo [25–29] and confirmed in preclinical studies [25].

In the present study, the phytoestrogen, glabridin, had the same beneficial effects on bone tissues as E_2 or genistein, at a lower concentration than genistein and other phytoestrogens, emphasizing the potential of phytoestrogens, such as isoflavans, to modulate bone disorders in post-menopausal women.

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