Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Steroid Biochemistry & Molecular Biology 91 (2004) 147–155
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Estrogen-like activity of licorice root constituents: glabridin and glabrene, in vascular tissues in vitro and in vivo

Dalia Somjen [a,*], Esther Knoll [a], Jacob Vaya [b], Naftali Stern [a], Snait Tamir [b]

[a] *Institute of Endocrinology, Metabolism and Hypertension, Tel-Aviv Sourasky Medical Center and The Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv 64239, Israel*
[b] *Laboratory of Natural Medicinal Compounds, Migal–Galilee Technological Center, Kiryat-Shmona 10200 and Tel-Hai Academic College, Israel*

Received 3 April 2003; accepted 9 April 2004

Abstract

Post-menopausal women have higher incidence of heart diseases compared to pre-menopausal women, suggesting a protective role for estrogen. The recently Women's Health Initiative (WHI) randomized controlled trial concluded that the overall heart risk exceeded benefits from use of combined estrogen and progestin as hormone replacement therapy for an average of five years among healthy postmenopausal US women. Therefore, there is an urgent need for new agents with tissue-selective activity with no deleterious effects. In the present study, we tested the effects on vascular tissues in vitro and in vivo of two natural compounds derived from licorice root: glabridin, the major isoflavan, and glabrene, an isoflavene, both demonstrated estrogen-like activities. Similar to estradiol-17β (E2), glabridin (gla) stimulated DNA synthesis in human endothelial cells (ECV-304; E304) and had a bi-phasic effect on proliferation of human vascular smooth muscle cells (VSMC). Raloxifene inhibited gla as well as E2 activities. In animal studies, both intact females or after ovariectomy, gla similar to E2 stimulated the specific activity of creatine kinase (CK) in aorta (Ao) and in left ventricle of the heart (Lv). Glabrene (glb), on the other hand, had only the stimulatory effect on DNA synthesis in vascular cells, with no inhibition by raloxifene, suggesting a different mechanism of action. To further elucidate the mechanism of action of glb, cells were pre-incubated with glb and then exposed to either E2 or to gla; the DNA stimulation at low doses was unchanged but there was abolishment of the inhibition of VSMC cell proliferation at high doses as well as inhibition of CK stimulation by both E2 and by gla. We conclude that glb behaved differently than E2 or gla, but similarly to raloxifene, being a partial agonist/antagonist of E2. Glabridin, on the other hand, demonstrated only estrogenic activity. Therefore, we suggest the use of glb with or without E2 as a new agent for modulation of vascular injury and atherogenesis for the prevention of cardiovascular diseases in post-menopausal women.

Keywords: Glabridin; Glabrene; Estradiol; Raloxifene; Aorta; Left ventricle of the heart; Vascular cells; Creatine kinase

1. Introduction

The organic extract of licorice roots, which shows low binding affinity to estradiol receptors (ER) [1], is known to contain isoflavans, isoflavene, chalcones (Fig. 1) such as glabridin, glabrol, glabrene, 3-hydroxyglabrol, 4′-*O*-methylglabridin (4′-*O*MeG), Phaseollinisoflavan, hispaglabridin A (hisp A), hispaglabridin B (hisp B), isoprenylchalcone derivative (IPC), formononetin, and isoliquitireginin chalcone (ILC) [2]. The licorice root is one of the richest sources of a unique subclass of the flavonoid family, the isoflavans [2]. Recently, we have demonstrated that glabridin (gla), which is the major compound of this class, and glabrene (glb), an isoflavene, have diverse estrogen-like activities [3,4]. The similarity of gla structure and lipophilicity to that of estradiol 17β (E2) encourage us to investigate the isoflavans subclass as possible candidates for estrogen mimetic. Several features are common to the structures of gla and E2 (Fig. 1); both have an aromatic ring substituted with hydroxyl group at 4 (gla) or 3 position (E2), with additional three fused rings of phenanthrene shape. Both molecules are relatively lipophilic, containing a second hydroxyl group, although not at the same position (17β in E2 and 2′ in gla).

Estrogen was reported to reduce the risk of cardiovascular disease [5–7]. The incidence of heart diseases among pre-menopausal women is low compared with males, whereas among post-menopausal women, incidence approaches that of males. This protective effect of estrogen

* Corresponding author. Tel.: +972 3 6973306; fax: +972 3 6974473.
E-mail address: dalias@tasmc.health.gov.il (D. Somjen).

0960-0760/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2004.04.003

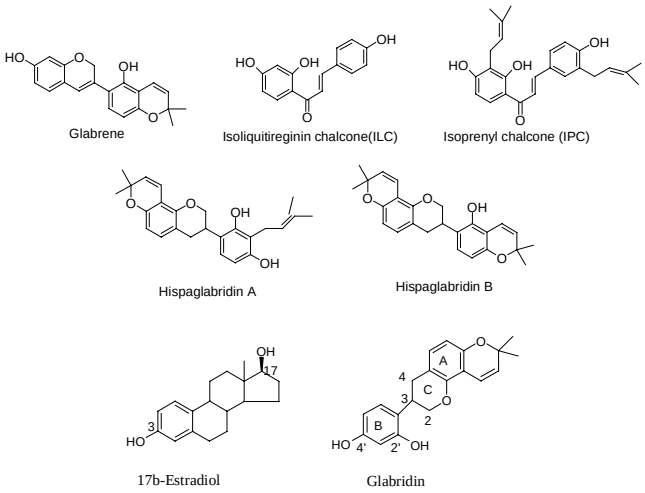

Fig. 1. The structure of phytoestrogens from licorice roots and estradiol 17β (E2).

may partially result from its effect on decreasing the ratio between LDL and HDL [8]. Administration of estrogen to postmenopausal women decreases the incident of heart diseases [9]. Recently, the principle results from Women's Health Initiative (WHI) randomized controlled trial were published concluding that overall heath risk exceeded benefits from use of combined estrogen and progestin for an average of five years among healthy postmenopausal US women. The increased risk means that in 10,000 women taking the drug for a year, there will be seven more coronary heart disease events, eight more invasive breast cancer, eight more strokes and eight more pulmonary emboli, but six fewer colorectal cancers and five fewer hip fractures [10]. These reports support the urgent need for new agents with no deleterious effects, which can replace the conventional hormone replacement therapy (HRT) treatments.

Animal and human studies indicate that estrogens are also protective against coronary atherosclerosis [5]. Because endothelial and vascular smooth muscle cells are involved in vascular injury and atherogenesis, the potential modulation of such processes by estrogen and estrogen-like compounds, such as glabridin and glabrene, is of obvious interest. In the present study, the effects of these compounds on the vascular system was tested in vitro and in vivo. The stimulation of creatine kinase (CK) specific activity in the left ventricle of the heart and in aorta, as well as CK activity and cell proliferation in human cultured vascular cells were measured. CK activity is known to be induced by estrogens in vivo and in vitro [11,12], and therefore can be used as an estrogen receptor (ER) response marker. In the present study, we found that both in vivo and in vitro glabridin was similar in its biological activity to estradiol E2 whereas, glabrene behaved differently. Moreover, glabrene also had characteristics of a phyto-SERM by having partial agonistic/antagonistic effects such as inhibiting the activity of E2. We therefore suggest that these licorice root-derived products can be used with or without estradiol as a new regime for hormone replacement therapy (HRT).

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]; Raloxifene (Evista®) was extracted from commercially available tablets.

2.2. Cell cultures

(a) Vascular smooth muscle cells (VSMC) containing both estrogen receptors α and β (14) were prepared from human umbilical artery, as previously described with minor modifications [13,14]. Cells were used only at passages 1–3 when expression of smooth muscle actin was clearly demonstrable.

(b) ECV-304 (E304), an endothelial cell line derived from a human umbilical vein containing both estrogen receptors α and β (14), was purchased from American Type Culture Collection (ATCC), Manassas, VA, USA and grown in medium 199 containing 10% FCS, glutamine and antibiotics.

2.3. Assessment of DNA synthesis

Cells were grown until subconfluence and then treated with various hormones or agents as indicated. Twenty-two hours following the exposure to these agents, [^{3}H] thymidine was added for 2 h. Cells were then treated with 10% ice-cold trichloroacetic acid (TCA) for 5 min and washed twice with 5% TCA and then with cold ethanol. The cellular layer was dissolved in 0.3 ml of 0.3N NaOH, samples were aspirated and [^{3}H] thymidine incorporation into DNA was determined [13–15].

2.4. Creatine kinase extraction and assay

Cells were treated for 4 h with the various agents as specified, scraped off and homogenized by freezing and thawing three times in an extraction buffer as previously described [13–15]. 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 (CK) was assayed by a coupled spectrophotometric assay described previously [13–15]. Protein was determined by Coomasie blue dye binding using bovine serum albumin (BSA) as the standard.

2.5. Animals

Immature female Wistar rats (25-day-old) were used as intact or two weeks post ovariectomy. The rats were housed in air-conditioned quarters with light from 5 to 19 h and

exposed to food and water ad libidum. All experiments were carried out according to the regulations of the Committee on Experimental Animals of the Tel-Aviv Sourasky Medical Center.

2.6. Creatine kinase extraction and assay

Changes in the specific activity of CK in aorta (Ao) and in left ventricle of the heart (Lv), induced by a single intraperitoneal injection (i.p.) of E2, gla or glb, were assayed in ovariectomized female rats, two weeks after surgery or in 25-day-old immature female rats ($n = 5$ per group for each experiment). Matched control rats ($n = 5$) were injected with vehicle: 0.05% ethanol in phosphate-buffer saline. E2 was injected at 5 μg for immature and 10 μg for ovariectomized female rats [12,16]. Rats were sacrificed 24 h after the injection. In multiple applications, rats were fed directly into the stomach daily for four days at the indicated doses. The organs examined were removed and stored at −20 °C until processed for assay of CK as described previously [12,16].

2.7. Statistical analysis

Differences between the mean values of experimental and the control groups were evaluated by analysis of variance (ANOVA).

3. Results

3.1. The effect of glabridin and glabrene on DNA synthesis in VSMC and E304 cells

When cells were incubated with increasing concentrations of gla or glb and DNA synthesis was measured, the following results were obtained. In VSMC, gla and glb at low concentrations stimulated DNA synthesis but at high concentrations only gla had a significant inhibitory effect. In E304 cells the dose-dependent stimulation of DNA synthesis was observed with both compounds (Fig. 2). The same biphasic effect was demonstrated using gla and glb on estrogen receptor (ER)-positive breast cancer cells [3,4] and was reported [17] for genistein. Neither E2 nor tamoxifen reversed the anti-proliferative effect, which support the hypothesis that the activities of phytoestrogens are mediated not only via the ER as E2 agonists but also at higher concentrations they act via other ER-independent cellular mechanisms, leading to inhibition of cell proliferation [17].

3.2. The effect of glabridin and glabrene on creatine kinase specific activity in VSMC and E304 cells

When cells were incubated with increasing concentrations of either gla or glb, there was a dose-dependent stimulation of CK specific activity in VSMC and in E304 cells by both agents (Fig. 3).

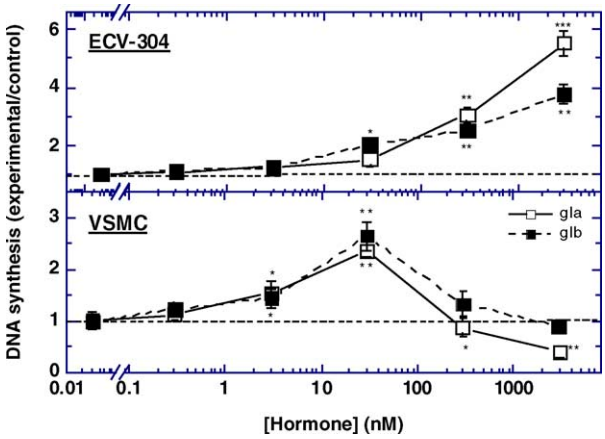

Fig. 2. The effect of glabridin (gla) and glabrene (glb) at 0.3–3000 nM on ^{3}[H] thymidine incorporation, in VSMC and in E304 cells. Cells were prepared, grown and hormonally treated and analyzed as described in the experimental section. Results are means ± S.E.M. of 4–12 incubates from 2–4 experiments and is expressed as the ratio between ^{3}[H] thymidine incorporation in hormone- and vehicle-treated (control) cells. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ for the comparison with control values. The statistical analysis was done by ANOVA. The basal levels of ^{3}[H] thymidine incorporation into DNA in VSMC and in E304 cells were 7200 ± 1080 and 94,100 ± 12,233 dpm/well, respectively.

3.3. Inhibition by raloxifene of the DNA synthesis and creatine kinase specific activity stimulated by glabridin or by glabrene in VSMC cells

When VSMC cells were incubated with gla or glb (30 or 3000 nM), alone or in the presence of raloxifene, a known

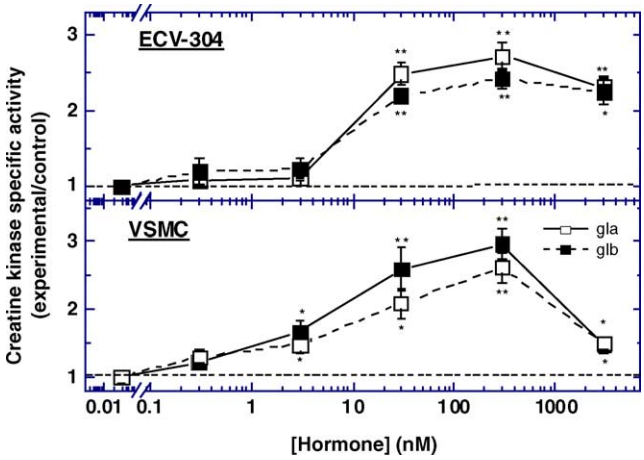

Fig. 3. The effect of glabridin (gla) and glabrene (glb) at 0.3–3000 nM on creatine kinase (CK) specific activity, in VSMC and in E304 cells. Cells were prepared, grown and hormonally treated and analyzed as described in the experimental section. Results are means ± S.E.M. of 4–12 incubates from 2–4 experiments and is expressed as the ratio between enzyme activity in hormone- and vehicle-treated (control) cells. $^*P < 0.05$; $^{**}P < 0.01$ for the comparison with control values. The statistical analysis was done by ANOVA. The basal levels of creatine kinase specific activity in VSMC and in E304 cells were 0.050 ± 0.001 and 0.116 ± 0.017 μmols/min/mg protein, respectively.

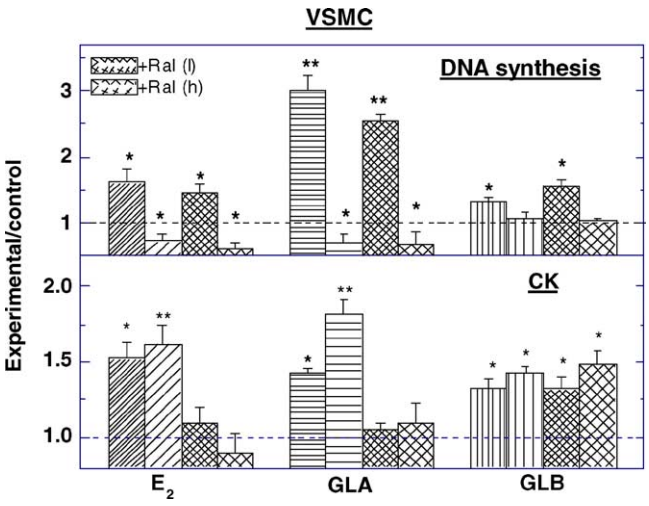

Fig. 4. Effects of raloxifene (Ral) (30 and 3000 nM) on the effects of E2 (0.3 and 30 nM) and glabridin (GLA) (30 and 3000 nM) or glabrene (GLB) (30 and 3000 nM) on ^{3}[H] thymidine incorporation and on creatine kinase specific activity in VSMC cells. Cells were prepared, grown and hormonally treated and analyzed as described in the experimental section. Cells were incubated with either the hormones alone: E2 or GLA or GLB at the two concentrations or together with 100× excess of Ral (30 nM with the low concentrations and 3000 nM with the high concentrations). Results are means ± S.E.M. of 4–12 incubates from 2–4 experiments and is expressed as the ratio between ^{3}[H] thymidine incorporation or as the ratio between enzyme activity in hormone- and vehicle-treated (control) cells. $^{*}P < 0.05$; $^{**}P < 0.01$ for the comparison with control values. The statistical analysis was done by ANOVA. Basal levels are the same as in legends to Fig. 1 and Fig. 2.

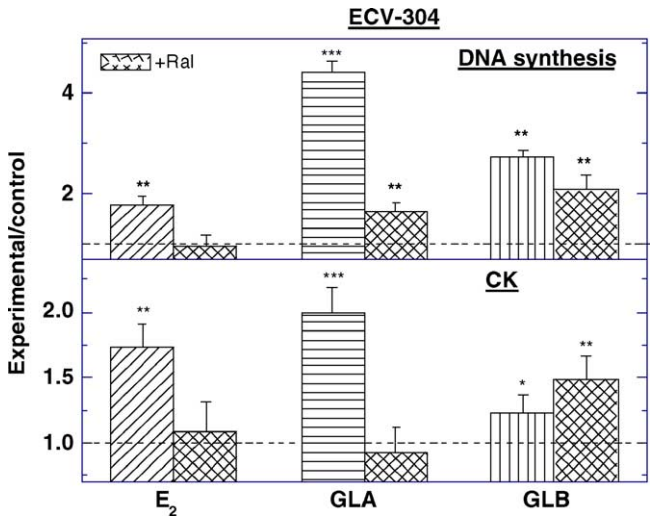

Fig. 5. Effects of raloxifene (Ral) (3000 nM) on the effects of E2 (30 nM) and glabridin (GLA) (3000 nM) or glabrene (GLB) (3000 nM) on ^{3}[H] thymidine incorporation and on creatine kinase specific activity in E304 cells. Cells were prepared, grown and hormonally treated and analyzed as described in the experimental section. Results are means ± S.E.M. of 4–12 incubates from 2–4 experiments and is expressed as the ratio between ^{3}[H] thymidine incorporation or as the ratio between enzyme activity in hormone- and vehicle-treated (control) cells. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ for the comparison with control values. The statistical analysis was done by ANOVA. Basal levels are the same as in legends to Fig. 1 and Fig. 2.

estrogen antagonist (RAL at 30 or 3000 nM), the stimulatory effects of E2 and GLA on CK were inhibited completely by Ral, but the stimulation of CK by GLB, was not inhibited by Ral (Fig. 4). Raloxifene had no effect on DNA synthesis changes induced by gla, but inhibited the effects of glb (Fig. 4), suggesting that glb may act via an ER-independent mechanism.

3.4. Inhibition by raloxifene of the DNA synthesis and creatine kinase specific activity stimulated by glabridin or by glabrene in E304 cells

When E304 cells were incubated with gla or glb (3000 nM), alone or together with raloxifene (Ral 3000 nM), raloxifene blocked partially the stimulatory activity of gla on DNA synthesis and blocked only slightly the stimulation of DNA by glb (Fig. 5). On the other hand, the stimulatory effect of gla on CK was inhibited completely by RAL, but the stimulation of CK by glb, was not (Fig. 5).

3.5. The effect of pretreatment with glabrene on the acute hormonal modulations of DNA synthesis in VSMC and E304 cells

When cells were pre-incubated for three days by daily treatment with 300 nM glb and then treated for 24 h with either E2 (30 nM) or with gla or glb (3000 nM) and DNA synthesis was determined, in VSMC, there was a complete reversal of the inhibition of DNA (Fig. 6) by E2 and gla, and a slight increase in the stimulation by glb. In E304 cells, no significant changes were observed (Fig. 6).

3.6. The effect of pretreatment with glabrene on the acute hormonal modulations on creatine kinase specific activity in VSMC and E304 cells

When cells were pre-incubated for three days by daily treatment with 300 nM glb and then treated for 4 h with either E2 (30 nM) with gla or glb (3000 nM) and CK was determined, there was up-regulation of the stimulation of CK activity by glb but no change in the response to E2 and to gla in VSMC (Fig. 7). On the other hand the response to E2 and gla was completely blocked, and no significant effects of the response to glb in E304 cells (Fig. 7), suggesting that glb acts as antagonist to E2 and gla in these cells.

3.7. The effect of pretreatment with non-hypercalcemic Vitamin D analog CB1093 on the acute hormonal modulations on DNA synthesis in VSMC and E304 cells

When VSMC cells were pre-treated for three days by daily addition of 1 nM CB1093 (CB), a non-hypercalcemic

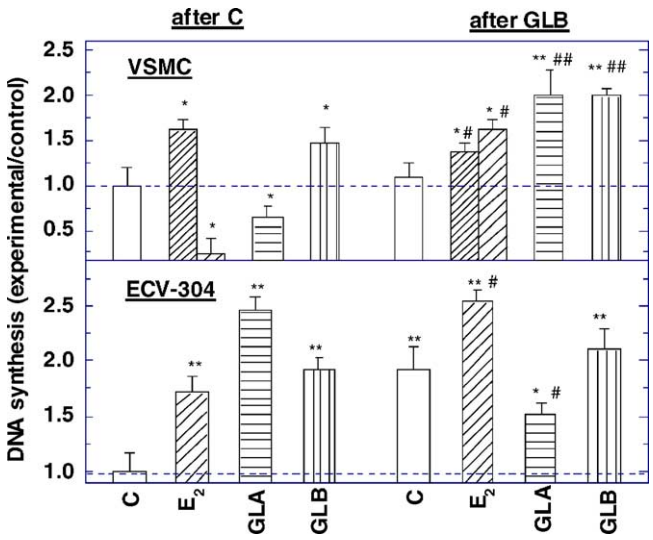

Fig. 6. Effects of pretreatment with daily additions for three days with glabrene (GLB 300 nM) on the effects of E2 (0.3 and 30 nM) and glabridin (gla) (3000 nM) or glabrene (glb) (3000 nM) on ^{3}[H] thymidine incorporation in VSMC and on the effects of E2 (30 nM) and glabridin (GLA) (3000 nM) or glabrene (GLB) (3000 nM) on ^{3}[H] thymidine incorporation in E304 cells. Cells were prepared, grown and hormonally treated and analyzed as described in the experimental section. Results are means ± S.E.M. of 4–12 incubates from 2–4 experiments of the ratio between ^{3}[H] thymidine incorporation in hormone- and vehicle-treated (control) cells. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ for comparison with control values, and $\#P < 0.05$; $\#\#P < 0.01$ for comparison with control values of GLB treatment. The statistical analysis was done by ANOVA. Basal levels are the same as in legends to Fig. 1 and Fig. 2.

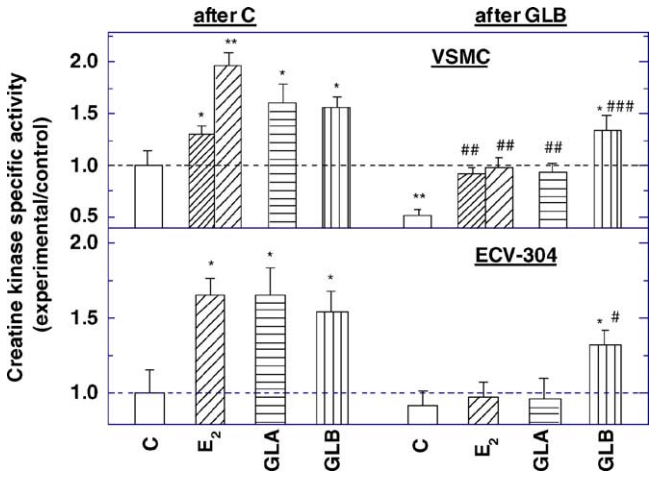

Fig. 7. Effects of pretreatment with daily additions for three days with glabrene (GLB 300 nM) on the effects of E2 (0.3 and 30 nM) and glabridin (GLA) (3000 nM) or glabrene (GLB) (3000 nM) on creatine kinase specific activity (CK) in VSMC and on the effects of E2 (30 nM) and glabridin (gla) (3000 nM) or glabrene (glb) (3000 nM) on creatine kinase (CK) specific activity in E304 cells. Cells were prepared, grown and hormonally treated and analyzed as described in the experimental section. Results are means ± S.E.M. of 4–12 incubates from 2–4 experiments of the ratio between enzyme activity in hormone- and vehicle-treated (control) cells. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ for comparison with control values, and $\#P < 0.05$; $\#\#P < 001$; $\#\#\#P < 0.001$ for comparison with control values of GLB treatment. Statistical analysis was done by ANOVA. Basal levels are the same as in legends to Fig. 1 and Fig. 2.

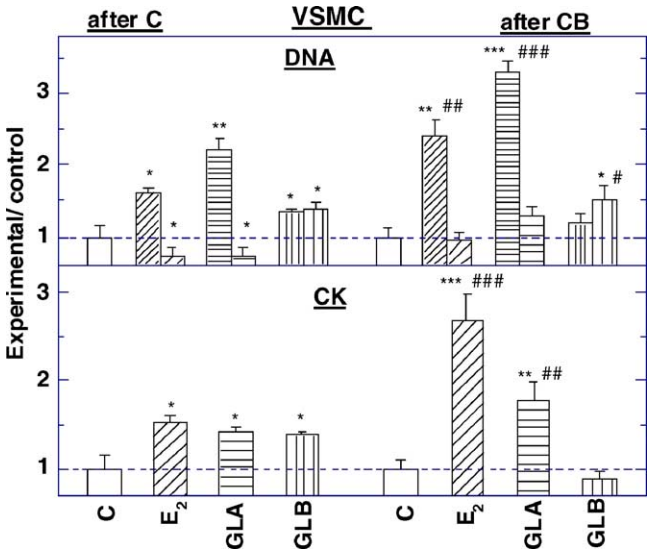

Fig. 8. Effects of pretreatment with daily additions for three days of the Vitamin D non-hypercalcemic analog CB1093 (CB at 1 nM) on the effects of E2 (0.3 and 30 nM) and glabridin (GLA) (30 and 3000 nM) or glabrene (GLB) (30 and 3000 nM), on ^{3}[H] thymidine incorporation and on the effects of E2 (30 nM) and glabridin (gla) (3000 nM) or glabrene (glb) (3000 nM), on creatine kinase (CK) specific activity in VSMC cells. Cells were prepared, grown and hormonally treated and analyzed as described in the experimental section. Results are means ± S.E.M. of 4–12 incubates from 2–4 experiments and are expressed as the ratio between ^{3}[H] thymidine incorporation and enzyme activity in hormone- and vehicle-treated (control) cells. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ for comparison with control values, and $\#P < 0.05$; $\#\#P < 0.01$; $\#\#\#P < 0.001$ for comparison with control values of CB treatment. Statistical analysis was done by ANOVA. Basal levels are the same as in legends to Fig. 1 and Fig. 2.

Vitamin D analog which affect ERα and ERβ expression, and then treated for 24 h with either E2 (0.3 or 30 nM), with gla or glb (30 or 3000 nM), and DNA synthesis was determined, there was an increase in the stimulation of DNA synthesis by low E2 or low gla, but inhibition of the stimulation of DNA by low concentration of glb (Fig. 8). On the other hand, the inhibition of DNA synthesis by high concentration of either E2 or gla was abolished, while no change with glb after pretreatment with CB (Fig. 8). The effect of CB on the DNA response in E304 to E2 or gla was unchanged while a decreased response was observed with glb on DNA (Fig. 9).

3.8. The effect of pretreatment with non-hypercalcemic Vitamin D analog CB1093 on the acute hormonal modulations on creatine kinase activity in VSMC and E304 cells

When VSMC were pre-incubated for three days by daily treatment with 1 nM CB 1093 (CB) and then treated for 24 h with either E2 (30 nM) with gla or glb (3000 nM) and CK was determined, there was upregulation of the stimulation of CK by E2 or gla but the response to glb was completely

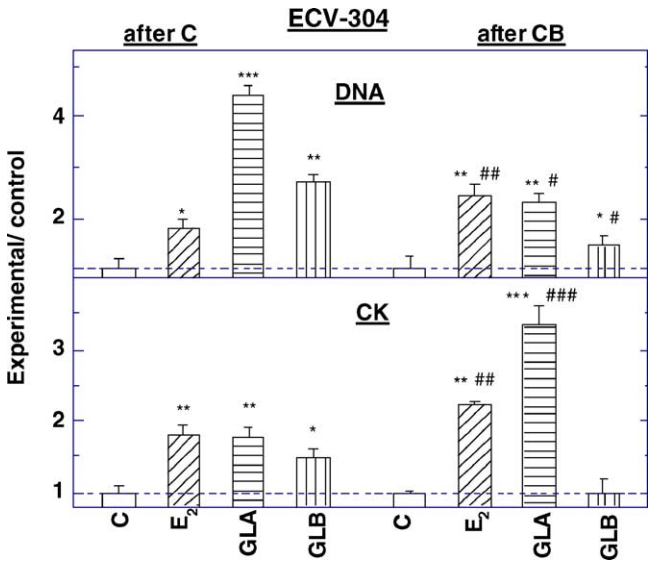

Fig. 9. Effects of pretreatment with daily additions for three days with the Vitamin D non-hypercalcemic analog CB1093 (CB at 1 nM) on the effects of E2 (30 nM) and glabridin (GLA) (3000 nM) or glabrene (GLB) (3000 nM) on ^{3}[H] thymidine incorporation and on creatine kinase (CK) specific activity in E304 cells. Cells were prepared, grown and hormonally treated and analyzed as described in the experimental section. Results are means ± S.E.M. of 4–12 incubates from 2–4 experiments and are expressed as the ratio between ^{3}[H] thymidine incorporation and enzyme activity in hormone-treated and control cells. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ for comparison with control values, and $\#\#P < 0.01$; $\#\#\#P < 0.001$ for comparison with control values of CB treatment. Statistical analysis was done by ANOVA. Basal levels are the same as in legends to Fig. 1 and Fig. 2.

inhibited (Fig. 8). Similar results were obtained with E304 cells (Fig. 9).

3.9. Stimulation of creatine kinase specific activity by a single injection of glabridin or glabrene into immature female rats

Immature female rats, weighing about 60 g, were injected with increasing concentrations of gla or glb (ranging from 3 to 300 μg/rat). Both compounds were tested for their effects on the specific activity of CK in rat vascular tissues: left ventricle of the heart (Lv), which contains vascular smooth muscle cells, and aorta (Ao), which contains endothelial cells (Fig. 10). Stimulation of CK by gla or glb in both organs was maximal at 300 μg, with a significant increase relative to vehicle-treated controls evident already by 30 μg (Fig. 10). Both compounds show agonistic behavior.

3.10. Inhibition by raloxifene of creatine kinase specific activity stimulated by glabridin or glabrene or by estradiol in immature female rats

Immature rats were injected with gla or glb at the optimal dosage (300 μg) or with E2 (5 μg), alone or together with

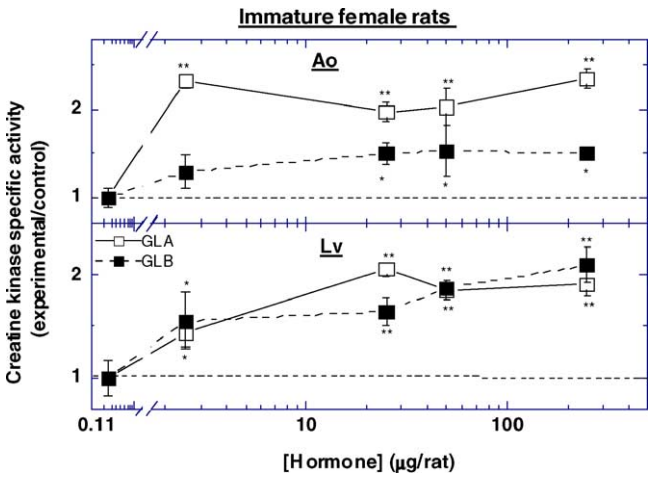

Fig. 10. The effect of glabridin (GLA) and glabrene (GLB) at 2.5–250 μg on creatine kinase (CK) specific activity in aorta (Ao) and left ventricle of the heart (Lv) of immature female rats. Extracts were prepared, and assayed as described in the experimental section. Results are means ± S.E.M. of $n = 9$ from three experiments and are expressed as the ratio between enzyme activity in hormone- and vehicle-treated (control) organs. $^{*}P < 0.05$; $^{**}P < 0.01$ for comparison with control values. Statistical analysis was done by ANOVA. The basal levels of CK in Ao and in Lv were 0.59 + 0.15 and in Lv 0.91 + 0.29 μmols/min/mg protein, respectively.

0.5 mg raloxifene (Ral). Raloxifene completely blocked the stimulatory activity of both gla and E2 on CK in Lv but only partially in Ao (Fig. 11). On the other hand, RAL in either Lv or Ao (Fig. 11) did not block the effects of glb, supporting the in vitro data indicating that glb may act via ER-independent mechanism.

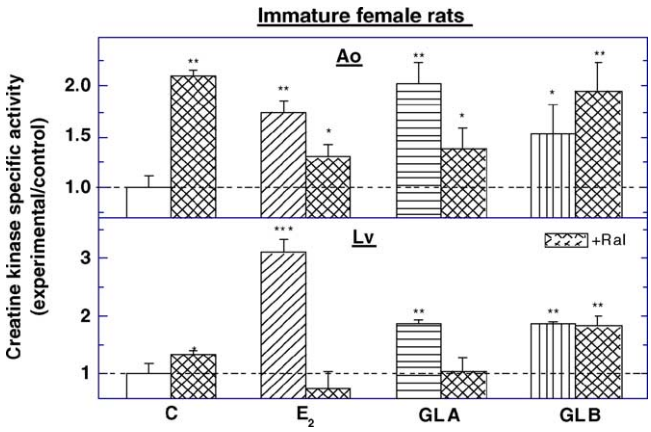

Fig. 11. The effect of raloxifene (Ral) (3000 nM) on the effects of estradiol (E2) (5 μg), glabridin (GLA) and glabrene (GLB) at 250 μg on creatine kinase (CK) specific activity in aorta (Ao) and left ventricle of the heart (Lv) of immature female rats. Extracts were prepared and assayed as described in the experimental section. Results are means ± S.E.M. of $n = 9$ from three experiments and are expressed as the ratio between enzyme activity in hormone- and vehicle-treated (control) organs. $^{*}P < 0.05$; $^{**}P < 0.01$ for comparison with control values. Statistical analysis was done by ANOVA. The basal levels of CK in Ao and in Lv were 0.79 + 0.15 and in Lv 0.66 + 0.29 μmols/min/mg protein, respectively.

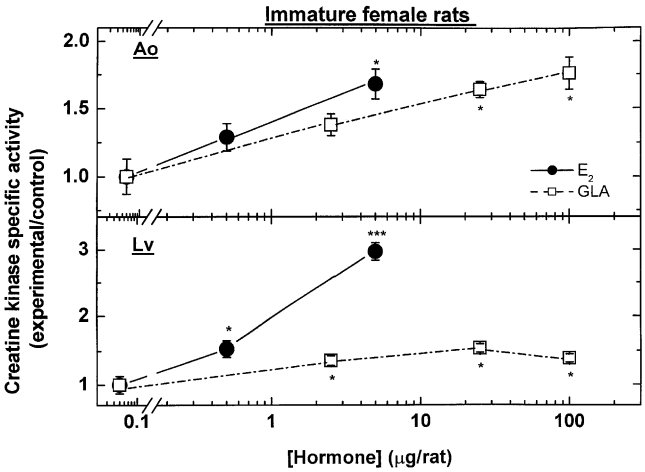

Fig. 12. The effect of E2 (0.5–5 μg) or glabridin (GLA) at 2.5–100 μg administered orally for three days on creatine kinase (CK) specific activity in aorta (Ao) and left ventricle of the heart (Lv) of immature female rats. Extracts were prepared, and assayed as described in the experimental section. Results are means ± S.E.M. of $n = 8$ from two experiments and are expressed as the ratio between enzyme activity in hormone- and vehicle-treated (control) organs. $^{*}P < 0.05$; $^{***}P < 0.001$ for comparison with control values. Statistical analysis was done by ANOVA. The basal levels of CK in Ao and in Lv were 0.79 + 0.17 and in Lv 0.65 + 0.29 μmols/min/mg protein, respectively.

3.11. Stimulation of creatine kinase specific activity by repeated oral administration of glabridin or estradiol in immature female rats

Daily feeding of gla (at concentrations of 2.5–250 μg/rat/day) or E2 (0.5–5 μg) for three days stimulated CK specific activity in Lv and in Ao (Fig. 12). The effects on Ao were much stronger than in Lv. When the daily feeding with 2.5 μg/day of E_2 or 25 μg/day of gla or both was extended for two weeks, the effects of gla were the same as those of E_2 in Ao, and both were additive (Table 1). In Lv, the effects were lower than in Ao. In Lv, gla was more effective than E_2 and was not significantly different than the mixture (Table 1).

Table 1
Stimulation of creatine kinase specific activity by repeated oral administration of glabridin, estradiol or both in immature female rats

	Aorta	Left ventricle of the heart
C	1.00 ± 0.06	1.00 ± 0.11
E2	1.50 ± 0.10*	1.37 ± 0.13*
Gla	1.38 ± 0.10*	1.78 ± 0.17**
Gla + E2	2.29 ± 0.13**	2.08 ± 0.16**

The effect of E2 (0.5 μg) or glabridin (Gla at 25 μg) or both administered orally for two weeks on creatine kinase (CK) specific activity in aorta (Ao) and left ventricle of the heart (Lv) of immature female rats. Extracts were prepared and assayed as described in the experimental section. Results are means ± S.E.M. of eight incubates from two experiments, expressed as the ratio between enzyme activity in hormone- and vehicle-treated (control) organs. $^{*}P < 0.05$; $^{**}P < 0.001$ for the comparison with control values. The statistical analysis was done by ANOVA. The basal levels of CK in Ao and in Lv were 0.73 + 0.07 and 0.65 + 0.29 μmols/min/mg protein, respectively.

Table 2
Stimulation of creatine kinase specific activity by glabridin or estradiol single injection in ovariectomized female rats

	Aorta	Left ventricle of the heart
C	1.00 ± 0.16	1.00 ± 0.22
E2	2.00 ± 0.19**	1.59 ± 0.10**
Gla	1.65 ± 0.19**	1.51 ± 0.16**

Effects of a single injection of E2 (10 μg) or Gla (250 μg) on creatine kinase (CK) specific activity in aorta (Ao) and in the left ventricle of the heart (Lv) of ovariectomized female rats. Details for treatment, extraction and analysis are given in Materials and methods. Results are means ± S.E.M. of eight assays from two experiments and are expressed as the ratios between the specific activities of CK in hormone-treated and control animals. $^{**}P < 0.01$. The basal activities of creatine kinase were in Ao and Lv 0.39 + 0.05 and 1.21 + 0.39 μmols/min/mg protein, respectively.

3.12. Stimulation of creatine kinase specific activity by glabridin or estradiol single injection in ovariectomized female rats

Ovariectomized female rats were injected with gla at the optimal concentration for adult rats (600 μg) or with E2 (10 μg) and the activity of CK in Lv and in Ao were determined (Table 2). In both organs, the effect of a single injection of gla was similar to that of a single injection of E2 (Table 2).

4. Discussion

Among the licorice constituents isolated and tested, the most active phytoestrogen in vitro and in vivo is glabridin (gla) [3,4,18]. Several features are common to the structures of gla and E2. Both have an aromatic ring substituted with a hydroxyl group at para (glabridin) or position 3 (estradiol), with three additional fused rings of a phenanthrenic shape. Both are relatively lipophilic, containing a second hydroxyl group (17β in estradiol and 2′-OH in glabridin). In the present study, the effects of gla on vascular cells were found to be similar to that of E2. Glabrene (glb), an isoflavene from the licorice roots containing double bond in ring C, although it is known to bind estrogen receptor and to stimulate growth of ER-positive human breast cancer cells [4] as other phytoestrogens, had different effects on vascular cells. The effect of gla in comparison to E2 was studied on E304 and VSMC, which are both, involved in vascular injury and atherogenesis processes, modulated by estrogen. Similar to E2, gla stimulated endothelial DNA synthesis and had a bi-phasic effect on proliferation of VSMC. Thus, the inhibition of VSMC proliferation and the induction of E304 cell proliferation by either E2 or gla are beneficial in preventing atherosclerosis [13]. To further characterize the estrogen-like activity of gla on vascular cells, treatment to-

gether with raloxifene, a known partial agonist/antagonist of estrogen, had the same inhibitory effects on both agents. Glabridin also had the same effect on the stimulation of DNA synthesis in vascular cell as treatment with genistein containing carboxymethyl ether at position 6 instead of the hydroxyl group (CG) also showed estrogenic activity in vivo and in vitro. It shows mixed agonistic/antagonistic activity on ERα; it blocked both the stimulatory and the inhibitory effects of E2 on DNA in VSMC. It blocked CK induced by E2 in both cell types and DNA synthesis in E304. CG behaved like raloxifene in rat organs in vivo, and from modeling, it binds to ERβ and blocks ERα [19]. These similarities in activity may result from similar mechanism of action, yet to be tested.

In animals studies gla stimulated CK (an early 'estrogen-induced protein' in vivo and in vitro) which is a known estrogen response marker [12] in Ao and in Lv similar to E2, when either injected to immature or ovariectomized female rats, or when fed to rats daily for three days. The data suggest that gla has estrogen-like activity in vascular tissues. Glabrene, on the other hand, has the same effects as gla on the DNA synthesis in vascular cells tested, but might also have an additional mechanism of action. In an attempt to understand glb activities, cells were pre-incubated with glb, and then exposed to either E2 or gla in VSMC, the DNA response at low doses was unchanged but there was abolishment of the inhibition of cell proliferation at high doses. This pretreatment inhibited CK stimulation by E2 and by gla in both cells. Glabrene showed similar effects to that of raloxifene on CK activity, which may suggest that glb is an antagonist to E2 in these processes, namely, like RAL it has both agonistic and antagonistic effects. Pretreatment with the Vitamin D analog CB1093 (CB) also blocked the inhibitory effect on DNA by either E2 or gla in VSMC and upregulated their stimulatory effects, with no change in the activity of glb. CB also upregulated CK response to E2 and gla in these cells and blocked the stimulation by glb. In E304 cells, CB increased the stimulation of both E2 and gla on DNA synthesis and on CK activity, but blocked both effects of glb. Thus, glb shows estrogen agonist and antagonist activities, which depend on the cell type, and the specific response marker tested, therefore, might be considered as a new SERM. The changes in the responses to gla or glb of the cells after vitamin D, probably reflects changes in ERα and ERβ as was shown before [14].

Animal and human studies indicate that estrogens are protective against coronary atherosclerosis [5] and observational studies reported a reduction in cardiovascular heart diseases (CHD) of 50% in postmenopausal women receiving HRT as compared with nonusers [9]. Recently controversies on cardiovascular risk in HRT treated women were reported in the Women's Health Initiative (WHI) trial,which found no overall reduction in risk of coronary heart disease events among postmenopausal women with CHD (10). However, in the hormone group, findings did suggest a higher risk of CHD events during the first year and a lower risk during 3–5 years. This trial increased the need for alternative hormone replacement therapy to postmenopausal women thus, the potential of estrogen-like compounds such as isoflavans to modulate vascular injury and atherogenesis is of obvious interest.

References

[1] J. Liu, J.E. Burdette, H. Xu, C. Gu, R.B. van Breemen, K.P. Bhat, N. Booth, A.I. Constantinou, J.M. Pezzuto, H.H. Fong, N.R. Farnsworth, J.L. Bolton, Evaluation of estrogenic activity of plant extracts for the potential treatment of menopausal symptoms, J. Agric. Food Chem. 49 (5) (2001) 2472–2479.
[2] J. Vaya, P.A. Belinky, M. Aviram, Antioxidant constituents from licorice roots: isolation, structure elucidation and antioxidative capacity toward LDL oxidation, Free Radic. Biol. Med. 23 (2) (1997) 302–313.
[3] S. Tamir, M. Eizenberg, D. Somjen, N. Stern, R. Shelach, A. Kaye, J. Vaya, Estrogenic and antiproliferative properties of glabridin from licorice in human breast cancer cells, Cancer Res. 60 (20) (2000) 5704–5709.
[4] S. Tamir, M. Eizenberg, D. Somjen, S. Izrael, J. Vaya, Estrogen-like activity of glabrene and other constituents isolated from licorice root, J. Steroid Biochem. Mol. Biol. 78 (3) (2001) 291–298.
[5] M.D. Iafrati, R.H. Karas, M. Aronovitz, S. Kim, T.R. Sullivan Jr., D.B. Lubahn, T.F. O'Donnell Jr., K.S. Korach, M.E. Mendelsohn, Estrogen inhibits the vascular injury response in estrogen receptor alpha-deficient mice, Nat. Med. 3 (5) (1997) 545–548.
[6] M. Seed, Sex hormones, lipoproteins, and cardiovascular risk, Atherosclerosis 90 (1) (1991) 1–7.
[7] L. Sourander, T. Rajala, I. Raiha, J. Makinen, R. Erkkola, H. Helenius, Cardiovascular and cancer morbidity and mortality and sudden cardiac death in postmenopausal women on oestrogen replacement therapy (ERT), Lancet 352 (9145) (1998) 1965–1969.
[8] D.A. Shewmon, J.L. Stock, C.J. Rosen, K.M. Heiniluoma, M.M. Hogue, A. Morrison, E.M. Doyle, T. Ukena, V. Weale, S. Baker, Tamoxifen and estrogen lower circulating lipoprotein (a) concentrations in healthy postmenopausal women, Arterioscler. Thromb. 14 (10) (1994) 1586–1593.
[9] M.J. Stampfer, G.A. Colditz, Estrogen replacement therapy and coronary heart disease: a quantitative assessment of the epidemiologic evidence, Prev. Med. 20 (1) (1991) 47–63.
[10] H.D. Nelson, L.L. Humphrey, P. Nygren, S.M. Teutsch, J.D. Allan, Postmenopausal hormone replacement therapy: scientific review, JAMA 288 (7) (2002) 872–881.
[11] S.D. Malnick, A. Shaer, H. Soreq, A.M. Kaye, Estrogen-induced creatine kinase in the reproductive system of the immature female rat, Endocrinology 113 (5) (1983) 1907–1909.
[12] D. Somjen, A. Waisman, J. Weisman, A.M. Kaye, Nonhypercalcemic analogs of Vitamin D stimulate creatine kinase B activity in osteoblast-like ROS 17/2.8 cells and up-regulate their responsiveness to estrogens, Steroids 63 (5/6) (1998) 340–343.
[13] D. Somjen, F. Kohen, A. Jaffe, Y. Amir-Zaltsman, E. Knoll, N. Stern, Effects of gonadal steroids and their antagonists on DNA synthesis in human vascular cells, Hypertension 32 (1) (1998) 39–45.
[14] D. Somjen, F. Kohen, Y. Amir-Zaltsman, E. Knoll, N. Stern, Vitamin D analogs modulate the action of gonadal steroids in human vascular cells in vitro, Am. J. Hypertens. 13 (4 Pt 1) (2000) 396–403.
[15] D. Somjen, E. Knoll, F. Kohen, N. Stern, Effects of phytoestrogens on DNA synthesis and creatine kinase activity in vascular cells, Am. J. Hypertens. 14 (12) (2001) 1256–1262.
[16] D. Somjen, Y. Weisman, A. Harell, E. Berger, A.M. Kaye, Direct and sex-specific stimulation by sex steroids of creatine kinase activity and DNA synthesis in rat bone, Proc. Natl. Acad. Sci. USA 86 (9) (1989) 3361–3365.

[17] D.T. Zava, G. Dauwe, Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells in vitro, Nutr. Cancer 27 (1) (1997) 31–40.
[18] S. Tamir, S. Izrael, J. Vaya, The effect of oxidative stress on ER alpha and ER beta expression, J. Steroid Biochem. Mol. Biol. 81 (4/5) (2002) 327–332.
[19] D. Somjen, Y. Amir-Zaltsman, B. Gayer, T. Kulik, E. Knoll, N. Stern, L.J. Lu, L. Toldo, F. Kohen, 6-Carboxymethyl genistein: a novel selective oestrogen receptor modulator (SERM) with unique, differential effects on the vasculature, bone and uterus, J. Endocrinol. 173 (3) (2002) 415–427.