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Mutual interaction of special phytoestrogenic compounds, their synthetic carboxy-derivatives and the less-calcemic vitamin D analog activities in human derived female cultured osteoblasts

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ABSTRACT

Cultured female-derived human bone cells (hObs) responded by different parameters to different phytoestrogenic and vitamin D compounds. Pre- and post-menopausal hObs express ERα and ERβ mRNA with higher abundance of ERα. Pre-treatment with the less-calcemic vitamin D analog JKF 1624F_2-2 (JKF) upregulated responsiveness to estrogens via modulation of ERs expression. These estrogenic compounds induce the expression and activity of 25 hydroxy-vitamin D_3-1α hydroxylase (1OHase). We now analyzed the effects of carboxy-genistein (cG), carboxy-biocainin A (cBA) and carboxy-daidzein (cD), of BA, D or G and of licorice derived compounds glabridin (Glb) and glabrene (Gla) and estradiol-17β (E_2) on DNA synthesis, creatine kinase specific activity (CK), intracellular and membranal E_2 binding and their modulations by JKF in hObs. We also analyzed modulation by phytoestrogenic compounds of 1OHase mRNA expression and activity. We showed that: (1) all compounds stimulated DNA synthesis and CK. (2) JKF and all estrogenic compounds modulated ERα and ERβ mRNA expression. (3) Pre-treatment with JKF increased DNA synthesis and CK responses only to E_2, D, G and Gla. (4) JKF increased the intracellular competitive binding only of E_2, D and G. (5) JKF abolished the membranal binding of all protein-bound estrogens. (6) JKF and all estrogenic compounds except the protein-bound ones up-regulated 1OHase expression and activity. In conclusion phytoestrogens and their analogs increase DNA synthesis and CK, and lead to increased production of 1,25$(OH)_2D_3$ in hObs, while pre-treatment with JKF modulates the effect of estrogenic compounds via regulation of ERs mRNA expression in a yet unclear mechanism.

1. Introduction

We have previously studied the effects of different estrogenic compounds including estradiol-17β (E_2) on bone in a rat model [1,2] using the increase in the specific activity of creatine kinase as a response marker. The brain type (BB) iso-enzyme of creatine kinase (CK) which is part of the "energy buffer" system, regulates the cellular concentration of ATP and ADP, is the major component of the "E_2-induced protein" of rat uterus [3] and is an efficient response marker to detect activity of E_2 as well as other estrogens, in bone cells *in vivo* and *in vitro* [1,4], which express low concentrations of E_2 receptors [5,6]. Notably, the stimulation of CK in cultured bone cells which correlated with the increased [^{3}H] thymidine incorporation into DNA (DNA synthesis) requires the higher end of the physiological range of E_2 concentrations [1,2].

Estrogen is well known for its beneficial effect in osteoporosis [7]. The biological effects of different estrogens are initiated by binding to the different estrogen receptors (ERs). Two major ERs have been identified: ERα and ERβ, which differ in their structure and tissue distribution [8]. New estrogenic compounds, which can replace estrogen itself with no such deleterious effects, are highly desirable [9].

In human-derived cultured bone cells (hObs), we found that E_2 increased cell proliferation and CK specific activity in a gender specific manner [10].

Phytoestrogens are heterogeneous group of plant-derived compounds some of which are selective estrogen receptor modulators (SERMs). All phytoestrogens are polyphenolic compounds with structural similarities to natural and synthetic estrogens; however they bind to the estrogen receptors with much lower affinity

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than E_2 itself [11]. Soybeans and soy foods are the most significant dietary sources of the isoflavone class of phytoestrogens, which includes genistein, daidzein and biochainin A [12,13] and have estrogenic activity on bone and the cardiovascular system but some of them have anti-estrogenic activity on breast cancer [13]. They have been proposed to prevent bone resorption and promote bone density [11–13].

Optimal bone growth and prevention of osteoporosis in post-menopausal women require adequate concentrations not only of estrogens but also of vitamin D_3 [14]. Pre-treatment with $1,25(OH)_2D_3$ (1,25) up-regulated sex-specific responsiveness and sensitivity to E_2 and to several SERMs in osteoblast-like cell lines (ROS 17/2.8 and SaOS2) and in rat bone, as measured by both the stimulation of CK and the increase in DNA synthesis [15,16]. This mutual interaction between E_2 and vitamin D was also manifested by an increase in ER after treatment with $1,25(OH)_2D_3$ [4]. However, the use of vitamin D is restricted due to its hypercalcemic effects. We reported that treatments with "less-calcemic" analogs of vitamin D, particularly JKF $1624F_2$-2 (JKF) [17] stimulated CK specific activity in ROS 17/2.8 osteoblast-like cells [18] and pre-treatment of skeletal-derived cells with these analogs, up-regulated responsiveness and sensitivity to E_2 [18,19].

In the present study, we measured responses of cultured human female osteoblast-like cells (hObs) to phytoestrogens to their synthetic carboxy-derivatives cG, cBA, cD, their protein-bound carboxy-derivatives cG-Ov, cD-Ov and cBA-BSA as well as the licorice derived phytoestrogens glabrene (Glb) and glabridin (Gla) and their modulated response by pre-treatment with JKF. We sought to correlate these response changes with the expression of the mRNA for ERα and for ERβ in the cells. We also analyzed the reciprocal effect of treatment with the estrogenic compounds on the vitamin D system; analyzing changes in both mRNA expression and enzymatic activity of 25 hydroxy vitamin D_3 $1-\alpha$ hydroxylase (1OHase) resulting in the synthesis of $1,25(OH)_2D_3$ in hObs leading to changes in its biology [20].

2. Materials and methods

2.1. Reagents

E_2, daidzein (D), genistein (G), biocainin A (BA) and CK assay kit were purchased from Sigma (St. Louis, MO). $JK1624F_2$-2 (JKF), carboxy-D (cD), carboxy-G (cG) and carboxy-BA (cBA) as well as their protein-bound derivatives and the licorice-derived products: glabridin (Glb) and glabrene (Gla) were synthesized by us [26,17]. All reagents were of analytical grade.

2.2. Cell cultures

Human female bone cells from pre- and post-menopausal women (hObs) were prepared from bone explants, by a non-enzymatic method as described previously [10]. 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 (PBS) to remove blood components. The explants were incubated in DMEM medium without Ca^{2+} (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. Hormonal treatment

Cells were pre-treated daily with vehicle (0.01% ethanol in medium) or JKF at 1 nM final concentration [18], for three days, starting on day 1 after seeding. On day 4 after seeding, the cultures were treated for 24 h with 30 nM E_2, cG, cD or cBA (300 nM) or 3 μM G, D, BA, Gla or Glb or protein-bound carboxy-phytoestrogens at 300 nM followed by harvesting for CK assay or DNA synthesis.

2.4. Creatine kinase extraction and assay in human female-derived 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 [10]. Supernatant extracts were obtained by centrifugation of homogenates at $14\,000 \times g$ for 5 min at 4 °C in an Eppendorf micro-centrifuge. Creatine kinase activity (CK) was assayed by a coupled spectrophotometric assay, as previously described [20,23]. Protein was determined by Coomasie blue dye binding, using bovine serum albumin (BSA) as the standard.

2.5. DNA synthesis in human female-derived osteoblasts

Cells were grown until sub-confluence 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 trichloro-acetic 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 0.3 N NaOH, samples were aspirated and [^{3}H] thymidine incorporation into DNA was determined [19].

2.6. Determination of mRNA for ERα and ERβ by real time PCR in human female-derived osteoblasts

RNA was extracted from cultured human bone cells, shown previously [19] to contain ERα and ERβ by Western blot analysis [4], and subjected to reverse transcription as previously described [29,31]. For ERα, we used 5 μl of cDNA in the reaction mixture with the primers 5′ AATTCTGACAATCGACGCCAG 3′ (forward) and 5′ GTGCTTCAACATTCTCCCTCCTC 3′ (reverse). For ERβ, the same amount of cDNA was used with the primers 5′ TGCTTTGGTTTGGGTGATTGC 3′ (forward) and 5′ TTTGCTTTTACTGTCCTCTGC 3′ (reverse). The reaction was carried out for 30 cycles at 94 °C for 30 s, at 58 °C for 30 s and at 72 °C for 1 min. ERα and ERβ cDNA were used as standard controls and compared to RNAse P as internal control for mRNA.

2.7. Competitive binding assay for intracellular estrogenic binding sites in human female-derived osteoblasts

Cells with and without pre-treatment with JKF, were incubated for 60 min at 37 °C with ^{3}[H] E_2 with and without excess of unlabelled compounds as described. Binding was terminated by four successive washes with ice-cold binding medium, and cellular content of ^{3}[H] E_2 was measured in a scintillation counter [19]..

2.8. Competitive binding assay for membranal estrogenic binding sites in human female-derived osteoblasts

Cells with and without pre-treatment with JKF, were incubated for 60 min at 37 °C with [Eu] E_2-BSA with and without excess of unlabelled protein-bound compounds as described. Binding was terminated by four successive washes with ice-cold binding medium, and cellular content of [Eu] E_2-BSA was measured in DELFIA counter [19].

2.9. Determination of mRNA for 25 hydroxy vitamin D_3 1 – α hydroxylase by real time PCR in human female-derived osteoblasts

Total RNA from cultured hOb was extracted using the Trizol Reagent (Gibco). An aliquot of 1 μg RNA from each sample was reverse transcribed (RT) using Advantage RT for PCR kit (Clonthec), as previously described [20]. 1OHase mRNA levels were analyzed using the ABI 7700 sequence detection system. Amplification of its cDNA was performed in 25 μl of the sample on 96 well plates in a reaction buffer containing Taqman universal PCR master mixture. The sequences of nucleotides were as follows: forward primers: CACCCGACACGGAGACCTT; reverse primers: TCAACAGCGTGGACACAAACA; Taqman probe: TCCGCGCTGTGGGCTCGG. RNAse P expression served as an internal control for each sample and was performed by an assay on demand gene expression products, which consist of a 20× mixture of unlabeled PCR primers and Taqman MGB probe labeled with 5′ carboxy fluorescein (FAM) dye. Measurements were performed in triplicates. The PCR conditions were: 50 °C for 2 min, 95 °C for 10 min, 50 cycles of 95 °C for 15 s and 60 °C for 1 min. The total volume of the reaction was 25 μl, 12.5 μl universal master mix, 1.25 μl 20× assay on demand mix and 11.25 μl cDNA [20].

2.10. Assessment of 25 hydroxy-vitamin D_3 1 – α hydroxylase activity in human female-derived osteoblasts

25 hydroxy vitamin D_3 1 – α hydroxylase activity was assessed by the measurement of 1,25 $(OH)_2D_3$ (1,25D) generated in hObs within 60 min after the addition of 25(OH)D_3 (200 ng/ml) to the culture, using the 1,25 ^{125}I RIA kit from Dia Sorin, MN, USA [20]. Protein of the cellular layer was assayed by the Bradford method.

2.11. 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. Expression and modulation by the phytoestrogenic compounds of ERα and ERβ in human derived-female osteoblasts

Female-derived human bone cells (hObs) from both ages expressed mRNA for both ERα and ERβ as measured by real time PCR. The ratio of ERα to ERβ was 121:1 in pre- and 78:1 in post-menopausal derived hObs. Pre-treatment with all phytoestrogenic compounds increased the expression of ERα in both age groups except treatment with BA which had no effect on post-menopausal hObs. The expression of ERβ was decreased in hObs from both age groups by G and cG as well as E_2, whereas cD had no effect, and cBA stimulated its expression. In pre-menopausal hObs, D and BA also inhibited ERβ expression, whereas in post-menopausal hObs, D stimulated this expression while BA had no effect (Fig. 1).

3.2. Expression and modulation by the protein-bound phytoestrogenic compounds of ERα and ERβ in human derived-female osteoblasts

Female-derived bone cells (hObs) from both ages expressed mRNA for both ERα and ERβ as measured by real time PCR. Pre-treatment with all phytoestrogenic compounds modulated the expression of ERα and ERβ in hObs from both age groups (Fig. 2). On the other hand all protein-bound phytoestrogenic compounds had no effect in hObs from both age groups on the expression of both ERα and ERβ (Fig. 2).

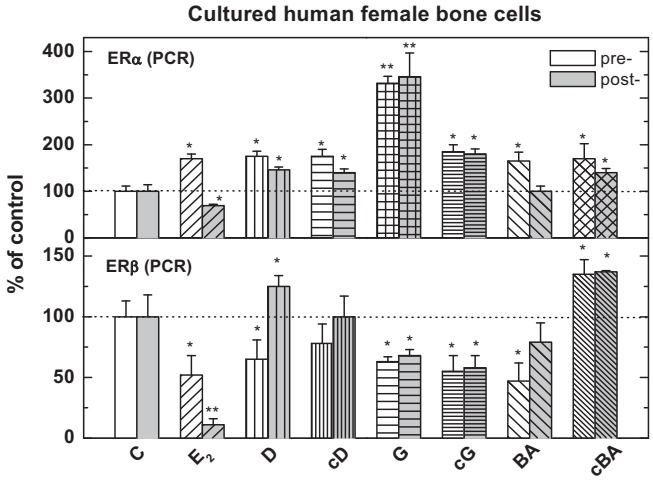

Fig. 1. Modulation of the expression of mRNA for ERα and ERβ in hObs from pre- and post-menopausal women, by pre-treatment with different phytoestrogens. Bone cells were obtained, cultured, treated daily for 3 days with E_2 30 nM, 300 nM cD, cG or cBA or 3 μM D, G or BA and 300 nM Gla or Glb and extracts prepared for real time PCR analysis as described in Section 2. Results are means ± SEM for triplicate cultures from 5 donors for each group. Means of hormone-treated cells were compared to means of vehicle-treated cells, $^{*}P < 0.05$; $^{**}P < 0.01$.

3.3. Pre-treatment with JKF modulated the stimulation of DNA synthesis by phytoestrogenic compounds in human derived-female osteoblasts

JKF which stimulated only slightly [^{3}H] thymidine incorporation in hObs from both age groups (12 and 15%, respectively), did not change the stimulation of DNA synthesis by cG, cD, cBA or Glb but did up regulate significantly ($P < 0.05$) the stimulation by G, D and Gla like E_2 in pre-menopausal hObs (Fig. 3). In post-menopausal hObs the results were similar except that the effect of Glb was significantly inhibited ($P < 0.05$, Fig. 3).

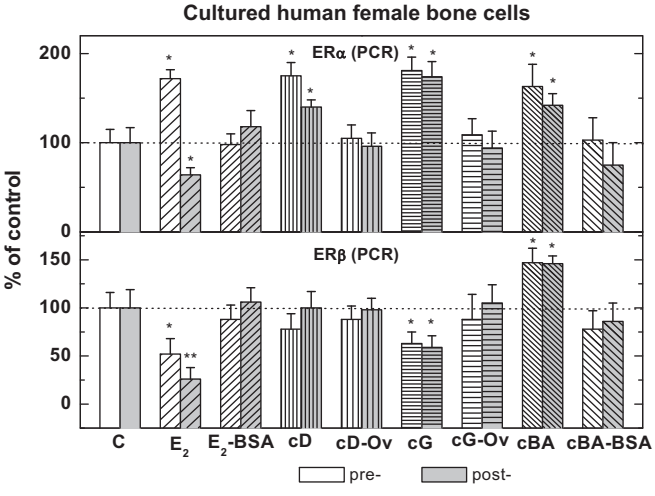

Fig. 2. Modulation by pre-treatment with different phytoestrogens and their protein-bound compounds of the expression of mRNA for ERα and ERβ in hObs from pre- and post-menopausal women. Bone cells were obtained, cultured, treated daily for 3 days with E_2 30 nM, 300 nM cD, cG or cBA or 300 nM protein-bound compounds and extracts prepared for real time PCR analysis as described in Section 2. Results are means ± SEM for triplicate cultures from 5 donors for each group. Means of hormone-treated cells were compared to means of vehicle-treated cells, $^{*}P < 0.05$; $^{**}P < 0.01$.

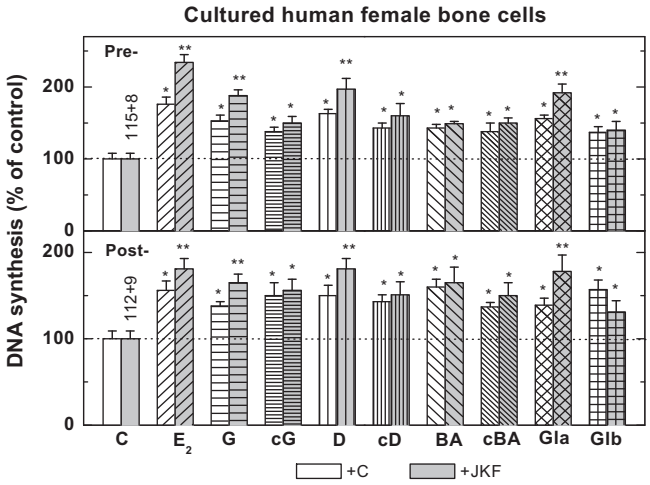

Fig. 3. Stimulation of DNA synthesis by different phytoestrogens in hObs from pre- and post-menopausal women after pre-treatment with JKF. Cells were obtained, cultured, treated and assayed for DNA synthesis as described in Section 2. They were treated for 3 days by daily addition of 1 nM JKF and then treated for 24 h with vehicle (C), 30 nM E_2, 300 nM cG, cD or cBA or 3 μM D, G, BA or 300 nM Gla or Glb. Results are means ± SEM for triplicate cultures from 5 women/group for JKF treated cultures and 10 women for control cultures. Control means were 6286 ± 675 and 5824 ± 460 dpm/well, for pre- and post-menopausal women respectively. Experimental means compared to control means: $^{*}P < 0.05$; $^{**}P < 0.01$.

3.4. Pre-treatment with JKF modulated the stimulation of CK specific activity by phytoestrogenic compounds in human female-derived osteoblasts

JKF which stimulated only slightly CK activity in hObs from both age groups (8 and 10%, respectively), up regulated significantly ($P < 0.05$) in pre-menopausal hObs only the stimulation of enzyme activity by G, D and Gla like E_2 (Fig. 4). In post-menopausal cells the modulation by JKF was similar except that there was significantly ($P < 0.05$) down regulation of the response to Glb (Fig. 3).

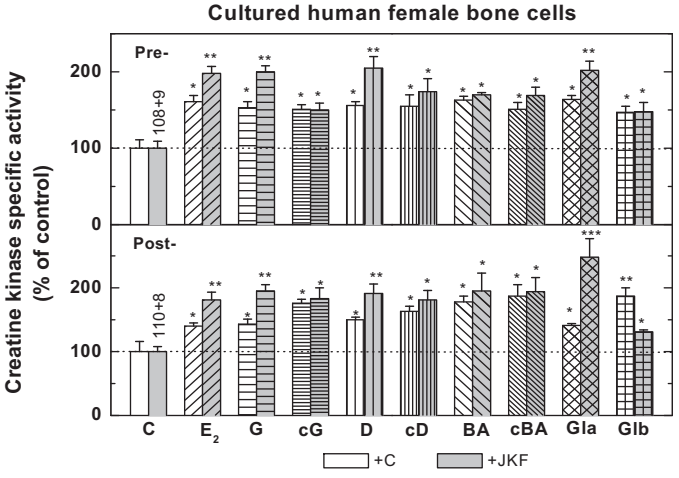

Fig. 4. Stimulation of CK specific activity by different phytoestrogens in hObs from pre- and post-menopausal women after pre-treatment with JKF. Cells were obtained, cultured, treated and assayed for CK activity as described in Section 2. Cells were treated for 3 days by daily addition of 1 nM JKF and then treated for 24 h with vehicle (C), 30 nM E_2, 300 nM cG, cD or cBA or 3 μM D, G, BA or 300 nM Gla or Glb. Results are means ± SEM for triplicate cultures from 5 women/group for JKF treated cultures and 10 women for control cultures. Control means were 28.6 ± 6.5 and 24.6 ± 4.0 nmol/min/mg protein, for pre- and post-menopausal women respectively. Experimental means compared to control means: $^{*}P < 0.05$; $^{**}P < 0.01$.

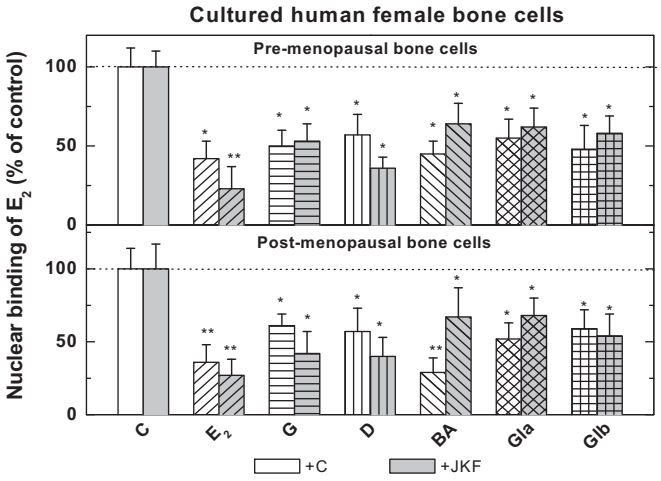

Fig. 5. The effect of pre-treatment with vitamin D_3 analog JKF on intracellular binding of phytoestrogens in pre- or post-menopausal female hObs. The intracellular binding of phytoestrogens was measured by competition of the binding of ^{3}[H] E_2. Results are means of 4 experiments and are expressed as % modulation of the binding in JKF treated cells compared to untreated cells. $^{**}P < 0.01$; $^{**}P < 0.01$.

3.5. Intracellular binding of E_2 and its modulation by JKF in human female derived-osteoblasts

Both pre- and post-menopausal female derived hObs demonstrated E_2 specific binding of ^{3}[H] E_2 (Fig. 5), presumably predominantly nuclear under these conditions (37 °C for 60 min). All estrogenic compounds tested for competition with ^{3}[H] E_2, like D, G, BA, Gla and Glb as E_2 itself showed significant binding in hObs from both age groups. Pre-treatment of hObs with JKF increased significantly ($P < 0.05$) the specific binding of ^{3}[H] E_2 in cells from both age groups. The specific binding of D or G, but not BA, Gla or Glb was significantly ($P < 0.05$) elevated in hObs from both age groups after JKF pre-treatment (Fig. 5). Of note is the fact that BA and Gla significantly ($P < 0.05$) attenuate the effect of E_2 (Fig. 5).

3.6. Membranal binding of E_2 and its modulation by JKF in human female derived-osteoblasts

Both pre- and post-menopausal female derived human bone cells demonstrated membranal E_2 specific binding of [Eu] E_2-BSA (Fig. 6). All estrogens bound to protein tested for competition with [Eu] E_2-BSA, like cG-Ov, cBA-BSA, and cD-Ov similar to E2-Ov showed significantly ($P < 0.05$) binding in both age groups. Pre treatment of hObs with JKF decreased significantly ($P < 0.05$) the specific binding of [Eu] E_2-BSA in cells from both age groups (Fig. 6).

3.7. Modulation by JKF of 25 hydroxy vitamin D_3 1 – α hydroxylase expression in human female-derived osteoblasts

Female-derived bone cells from both ages as well as male-derived cells expressed mRNA for 25 hydroxy vitamin D_3 1 – α hydroxylase (1OHase) as measured by real time PCR (Fig. 7). Pre treatment with JKF increased the expression of 1OHase in both age groups but not in male derived cells, with higher stimulation in pre-menopausal cells (Fig. 7).

3.8. Modulation by the phytoestrogenic compounds of 25 hydroxy vitamin D_3 1 – α hydroxylase expression in human female-derived osteoblasts

Female-derived bone cells from both ages expressed mRNA for 25 hydroxy vitamin D_3 1 – α hydroxylase (1OHase) as measured

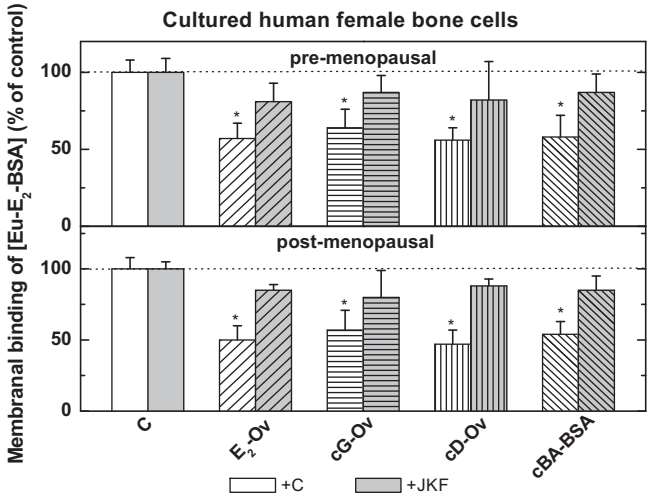

Fig. 6. The effect of pre-treatment with vitamin D_3 analog JKF, on the specific binding of phytoestrogens to membranal binding sites in pre- or post-menopausal female hObs. Bone cells were cultured, treated and assayed for membranal binding of Eu-BSA-E_2 as described in Section 2. Results are means ± SEM for % of specific binding of triplicate cultures in the presence of 500 fold concentration of the different phytoestrogenic-protein conjugates as competitors. Experimental means compared to control means: $^*P < 0.05$, $^{**}P < 0.01$.

by real time PCR (Fig. 8). Pre-treatment with all phytoestrogenic compounds, except BA in post-menopausal hObs, increased the expression 1OHase in both age groups (Fig. 8).

3.9. Modulation by the phytoestrogenic compounds of 25 hydroxy vitamin D_3 1 − α hydroxylase activity in human female-derived osteoblasts

Female-derived bone cells from both ages produce $1,25(OH)_2D_3$ as measured by radio immunoassay (Fig. 9). All free or carboxy-derivatives of the phytoestrogenic compounds stimulated the activity of 1OHase by increase the production of $1,25(OH)_2D_3$ (Fig. 9). The protein-bound phytoestrogenic compounds did not affect enzymic activity (Fig. 9).

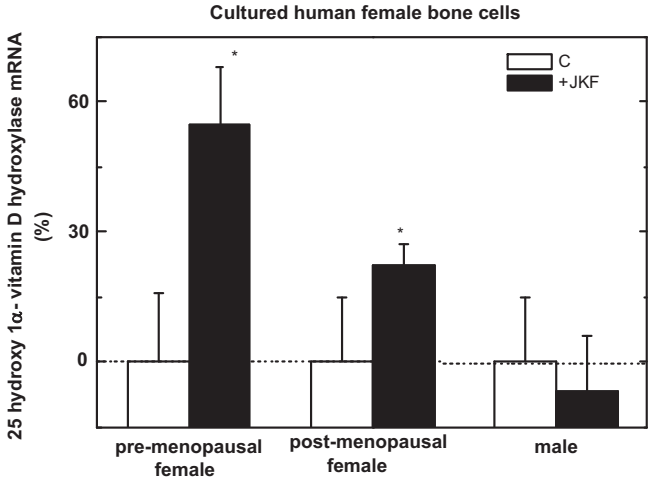

Fig. 7. Modulation of the expression of mRNA for 1OHase in hObs from pre- and post-menopausal women and male bone by pre-treatment with vitamin D_3 analog JKF. Bone cells were obtained, cultured, treated daily for 3 days with 1 nM JKF and extracts prepared for real time PCR analysis as described in Section 2. Results are means ± SEM for triplicate cultures from 5 donors for each group. Means of hormone-treated cells were compared to means of vehicle-treated cells, $^*P < 0.05$.

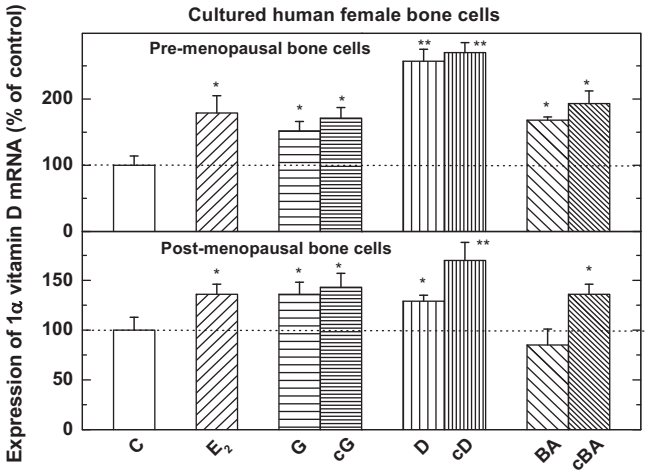

Fig. 8. Modulation of the expression of mRNA for 1OHase in hObs from pre- and post-menopausal women, by pre-treatment with different phytoestrogens. Bone cells were obtained, cultured, treated daily for 3 days with E_2 30 nM, 300 nM cD, cG or cBA and extracts were prepared for real time PCR analysis as described in Section 2. Results are means ± SEM for triplicate cultures from 5 donors for each group. Means of hormone-treated cells were compared to means of vehicle-treated cells, $^*P < 0.05$; $^{**}P < 0.01$.

4. Discussion

In our different studies including the present one, we found that the synthetic carboxy-derivatives of the phytoestrogens, E_2 and the free phytoestrogens stimulated age-dependently both DNA synthesis and CK specific activity in primary cultures of human female osteoblast-like cells (Figs. 3 and 4) [4,21,22].

Pre-treatment with JKF sharpens the difference between these phytoestrogenic compounds and E_2. Whereas the stimulation of D, G and Gla similar to E2 on both parameters and in both age groups was up-regulated, the effects of cG, cD, cBA and Glb were not significantly changed in either age group (Figs. 3 and 4). We showed that up-regulation of the stimulation by vitamin D_3 less-calcemic analogs occurs in normal human primary osteoblasts, accompanied by increase in ERα and decrease in ERβ. These changes in the ratio of

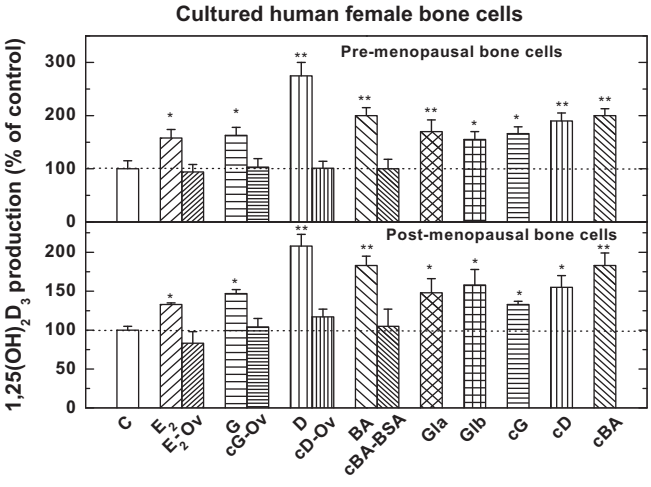

Fig. 9. Effects of phytoestrogens as well as their protein-bound derivatives on the production of 1,25D, in pre- and post-menopausal hObs. Cells were incubated for 24 h for the production of 1,25D with either E_2 (30 nM), cG (300 nM), cBA (300 nM), cD (300 nM), or D (3 μM), G (3 μM), BA (3 μM), Gla (300 nM) and Glb (300 nM) or 300 nM of the protein-bound hormones. Results are expressed as % change in the concentration of 1,25D (pg/mg protein), $^*P < 0.05$; $^{**}P < 0.01$, compared with control incubates containing the vehicle for the active compound only.

the different ERs to the favor of ERα may explain the modulations of the responses observed. We have previously demonstrated, using western blot analysis [4] that JKF increased both ERα and ERβ with a greater increase of ERα than ERβ in pre-menopausal-derived cells and the reverse in post-menopausal-derived cells [19]. Of note is that the free but not protein-bound estrogenic compounds are also modulating ERs expression in hObs (Fig. 2).

Whether or not our results are due to changes in binding to the different receptors is analyzed here. The effects of JKF on nuclear binding [29] are consistent with the increased responsiveness to D, G and Gla as E_2 after JKF pretreatment as well as changes in ER proteins (Fig. 5) [4]. In contrast the membranal binding of E_2 and all the phytoestrogens tested was abolished by JKF pretreatment (Fig. 6) [31]. Membranal binding is therefore not correlated with the up-regulation of DNA synthesis or CK specific activity stimulated by D, G and Gla as well as E_2 after JKF pretreatment. This indicates that membranal processes are not involved in CK stimulation by estrogenic compounds tested in this study or others (Fig. 6) [19,21,22].

While ERα and ERβ mRNA were found in both ages of female-derived bone cells, JKF increased ERα expression and decreased ERβ expression. On the other hand cG, cBA, D and E_2 down regulates significantly ERβ and increased ERα suggesting a negative effect of some estrogenic compounds on estrogenic responsiveness in cells from both age groups.

The synthesis of 1,25D from its precursor 25-hydroxyvitamin D_3 ($25(OH)D_3$), is catalyzed by 25 hydroxy vitamin D_3 $1-\alpha$ hydroxylase (1-OHase) in epithelial cells comprising various parts of the human nephron [23,24]. Renal 1-OHase is subject to tight systemic metabolic control by parathyroid hormone (PTH), calcium, phosphate and vitamin D_3 metabolites, predominantly 1,25D itself [25]. Renal 1-OHase is the major source of circulating 1,25D, which controls systemic calcium homeostasis; nevertheless, external-renal expression of 1-OHase and 1,25D is now well documented in various tissues and cell types such as prostate cells [24,26]. Molecular studies indicate that the enzyme is expressed in both the kidney and non-renal tissues [23,24] and is differentially regulated at least in some of these tissues [24,25]. In contrast to circulating 1,25D which controls systemic calcium homeostasis through its action on the intestinal mucosa, bone and kidney, accumulating data indicate that the 1,25D produced by extra-renal 1-OHase in various tissues does not contribute to circulatory levels but rather appears to act in an autocrine and/or paracrine fashion by modulating cell proliferation, differentiation, apoptosis, immunoregulation and other functions at a local level [27,28].

We have shown that human female osteoblasts express 1-OHase mRNA (Fig. 7) and is modulated by the phytoestrogenic compounds as by E_2. The cells also produced 1,25D which is modulated by different hormones including the phytoestrogenic compounds but not the protein-bound derivatives [20] (Fig. 9). The synthesis of 1,25D in hObs is quantitatively significant at basal production rate of ~1.5 pmol/mg protein/h, reaching ~4 pmol/mg protein/h under saturating concentrations of its substrate $25(OH)D_3$ [20], which might be important for the physiology of bone as was shown for other parameters.

The effects of these phytoestrogens as well as E_2 (Figs. 8 and 9) on 1-OHase expression and activity remain obscure. The current study demonstrates that all these compounds tested modulate local 1-OHase expression in hObs which might in turn modulate the effects of other estrogenic compounds on the cells (Figs. 3 and 4). It is not known however whether this happens also *in vivo*, if it does it might be used as a mixed new hormone replacement therapy for bone formation in post-menopausal osteoporosis.

The effect of the locally formed 1,25D might be involved in the modulatory effects of vitamin D on the hormonal responses of hObs to the different estrogenic compounds which might be due to the changes in the expression of estrogen receptors, which were reported to be changed with external vitamin D treatments as described previously (Figs. 1 and 2) [22,29]. This might be a defense mechanism in the patho-physiological conditions of absence of vitamin D in the body. Whether it suggests a differential interaction of phytoestrogens with the different binding sites through which it exerts its biological activity has still to be studied.

In conclusion, the present study provides evidence for the mutual interaction between special phytoestrogenic compounds and the vitamin D_3 system. The potential role of this system as an autocrine/paracrine mechanism to modulate bone cell metabolism and physiology warrants further investigation.

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