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Age-Dependent Responsiveness of Human Female Cultured Bone Cells to Estrogenic Compounds

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Abstract

Human cultured female osteoblasts (Obs) respond age-dependently to estradiol-17β (E2) and to phytoestrogens by increased DNA synthesis (DNA) and creatine kinase specific activity (CK). ERα mRNA expression is higher than ERβ mRNA in Obs. Pre-menopausal Ob (prOb) reveals higher expression of ERα than post-menopausal Ob (poOb), but similar intracellular and membranal E2 bindings. ERα mRNA is stimulated in prOb and inhibited in poOb by different estrogens. ERβ mRNA in prOb is stimulated by 2,3-bis (4-hydroxyphenyl)-propionitrile (DPN; ERβ specific agonist) and 4,4',4"-[4-Propyl-(1H)-pyrazol-1,3,5-triyl] tris-phenol (PPT; ERα specific agonist) and raloxifene (Ral) and inhibited by genistein (G) and daidzein (D) while in poOb only E2 and DPN inhibited it. All phytoestrogenic carboxy- derivatives stimulated ERα mRNA in both Ob, while the protein bound- carboxy were ineffective. All compounds except carboxy-biochainin A (cBA) had no effect on ERβ. There is higher expression of 1α 25 hydroxy- vitamin D (1OHase) mRNA in poOb, whereas $1,25(OH)_2D_3$ (1,25) production is similar, but all compounds increased 1OHase mRNA and 1,25 to higher extent in prOb. Obs express 15 and 12 lipoxygenase (12LO and 15LO) mRNA and produce 12 and 15 HETE (12H and 15H). 12LO expression is higher and 15LO is lower in prOb, while 12H is higher in prOb and 15H is similar in both. Both LOs are increased to higher extent in poOb by all estrogens except Ral and PPT. 12H is increased by DPN, PPT and carboxy- derivatives similarly in both Obs, while 15H is increased by biochainin A (BA), Ral and carboxy-derivatives. DNA synthesis and CK are stimulated by all compounds in both Obs, but to higher extent in prOb. The 1,25 less-calcemic analog JK 1624F2-2 (JKF) up-regulated DNA and CK response to all estrogens except BA and the carboxy- derivatives in both Obs. JKF stimulated intracellular binding of E2 similarly by all compounds except BA, but inhibited its membranal binding in both Obs. In conclusion Obs respond age-dependently to estrogens in a yet unknown mechanism and the beneficial outcome for human female bones is not yet clear.

Keywords: Osteoblasts; Estrogens; Vitamin D; ERs; LO: HETE

Introduction

Mammalian osteoblasts express specific intracellular and membranal receptors for estrogens [1-5] and respond to estrogens [6-11].

The age dependent- responses were studied by different researchers but still debatable, in human osteoblasts *in vitro* [12-17].

In our studies we analyzed the response of cultured female human derived osteoblasts (Ob) by different parameters to different estrogens, including estradiol 17β (E2), 2,3-bis (4-hydroxyphenyl)-propionitrile (DPN; ERβ specific agonist) and 4, 4′, 4" -[4-Propyl-(1H)-pyrazol-1,3,5-triyl] tris-phenol (PPT; ERα specific agonist), raloxifene (Ral) and phytoestrogens including their synthetic carboxy- derivatives and protein- bound carboxy- derivatives. Whether these findings imply also to human female bone response to hormonal treatments *in vivo* is yet not established.

We found that there is a higher expression of ERα mRNA and lower expression of ERβ mRNA in pre- than in post- menopausal Ob (prOb and poOb respectively), with no significant age difference in intracellular and membranal estrogen binding activities.

Ob also express of 1α 25 hydroxy vitamin D hydroxylase (1OHase) mRNA to higher extent in prOb compared to poOb, but no age-dependent difference in 1OHase activity namely 1,25(OH)2D3 (1,25) production. Ob express also 12 and 15 lipooxygenase (LO) mRNA and produce 12 and 15 HETE (H). 12LO mRNA is more expressed in prOb than poOb, but higher expression of 15LO mRNA in poOb than prOb. The production of 15H is the same in both, whereas the production of 12H is higher in prOb.

Human female derived cultured bone cells (Ob) show age-dependent changes in DNA synthesis (DNA) and CK specific activity (CK) in response to different estrogenic compounds [18].

The non- hypercalcemic vitamin D analog JK 1624F2-2 (JKF) up-regulated the response to E2 and some phytoestrogens in both Ob to the same extent.

The aim of the present review is to summarize our findings using cultured human female Ob derived from either pre- or post-menopausal women carried out in our laboratory on:

α. Modulation of ERα and ERβ mRNA expression.

β. Modulation of 1Ohase mRNA expression.

χ. Modulation of 1Ohase activity (1, 25 production).

δ. Modulation of 12LO and 15 LO mRNA expression.

ε. Modulation of 12LO and 15 LO activity 12H and 15H production.

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ϕ. Modulation by JKF of the DNA and CK response.

γ. Modulation by JKF of E2 binding both intracellular and membranal.

Materials and Methods

Reagents

CK assay kit, E_2, genistein (G), daidzein (D), biochainin A (BA) were from Sigma Chemicals Co. (St. Louis, MO). DPN and PPT were from Tocris biosciences (Bristol, BS11 OQl. UK). Ral was from Eli Lilly and Company. All other reagents were of analytical grade.

Carboxy derivatives and protein bound carboxy- derivatives of phytoestrogens were synthesized by us [19].

Cell cultures

Human female bone cells from pre- (<50 years old) and post-menopausal women (>55 years old) were prepared from bone explants obtained from biopsies of hip fractures from healthy patients, by a non-enzymatic method [14,20]. The bone-derived cells were identified as osteoblasts by major osteoblastic characteristics [13].

Hormonal treatment

1. Cultured cells were treated with control (vehicle; 0.01 % ethanol), 30nM E_2 or DPN (420nM) and PPT (390nM) or phytoestrogenic compounds or Ral (3000nM) for 24h, followed by harvesting either for CK preparation and assay, or for DNA [14,20].
2. Pre-treatment with JKF was performed by 3 daily additions of 1nM followed by 24h treatment with E2 (30nM) or different phytoestrogens or Ral (3000nM) as described [21].
3. Cultured cells were incubated for 1 hour with serum-free medium, followed by addition for 10 minutes of control (vehicle; 0.01 % ethanol), 30nM E_2 or 420nM DPN and 390nM PPT or 3000nM different phytoestrogens or 3000nM Ral followed by harvesting for 12 and 15 HETE extraction and assay [22].
4. Cultured cells were treated with control (vehicle;0.01 % ethanol), 30nM E_2 or 420nM DPN and 390nM PPT or 3000nM different phytoestrogens or Ral daily for three days, followed by harvesting for mRNA extraction and assay for different mRNA expression [18].

Creatine kinase preparation and assay

Cells were scraped off and homogenized by freezing and thawing and extracts were obtained for CK activity measurement [14,23]. Protein was assayed by Coomassie brilliant blue dye binding, using BSA as standard [24].

DNA synthesis assay

Cells were grown until sub- confluence, treated with control (vehicle; 0.01 % ethanol) and various hormones or agents as indicated. Twenty-two hours following the exposure to these agents, [^{3}H] thymidine was added for 2h. Cells were then treated with 10% ice-cold trichloro-acetic acid (TCA) for 5min and washed twice with 5% TCA and cold ethanol. The cellular layer was dissolved in 0.3ml of 0.3N NaOH, samples were aspirated and [^{3}H] thymidine incorporation into DNA was determined [14,23].

Determination of mRNA for ERα and ERβ or 1OHase or 12 and 15LO by real time PCR

RNA was extracted from cultured bone cells and subjected to reverse transcription. The reaction was carried out using ERα and ERβ or 1-OHase or 12LO and 15LO cDNA as standards, compared to RNAse P [22].

Determination of 1, 25 (OH)2D3 formation

1, 25 $(OH)_2D_3$ formation in cultured skeletal cells was assayed as previously described [25].

Determination of 12 or 15HETE formation

12 or 15HETE formation in cultured bone cells was assayed as described [25].

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. Binding was terminated by four successive washes with ice- cold binding medium, and cellular content of ^{3}[H] E_2 was measured [18].

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 [22] .

Statistical analysis

The significance of differences between experimental and control means was evaluated using Student's t-test in hich n=5 number of cultures.

Results

Age-dependent modulation of the expression of estrogen receptors in human female cultured bone cells

Human cultured bone cells derived from either prOb or poOb express both estrogen receptors (ERs); the ERα and ERβ mRNA with about 100 times higher abundance of ERα than ERβ in these cells (0.092+0.00075 compared to $0.0094+0.00012^{-\Delta CT}$ in prOb and 0.0075+0.00072 compared to $0.0104+0.0011^{-\Delta CT}$ in poOb). ERα mRNA expression in prOb is higher than in poOb (0.092+0.00075 compared to $0.0075+0.00072^{-CT}$) whereas ERβ is similar in both Ob (0.0094+0.00012 compared to $0.0104+0.0011^{-\Delta CT}$).

Three daily treatments of the different Obs with E2 or phytoestrogens resulted in modulation of the expression of both ERs (Figure 1a). ERα was stimulated in prOb by all compounds except the ERα agonist PPT, but inhibited in poOb by all compounds except D and PPT. On the other hand E2 and BA inhibited ERβ in both Ob, Ral, ERβ agonist DPN and PPT stimulated it in prOb whereas DPN inhibited it in poOb and D, G and Ral stimulated it in poOb whereas PPT had no effect (Figure 1a).

All carboxy derivatives of phytoestrogens up regulated ERα expression in both Ob to higher extent in prOb. On the other hand ERβ was stimulated in both Ob by cBA, inhibited by cG and no effect by cD (Figure 1b). All protein bound carboxy derivatives of phytoestrogens were not effective in any Ob (Figure 1b).

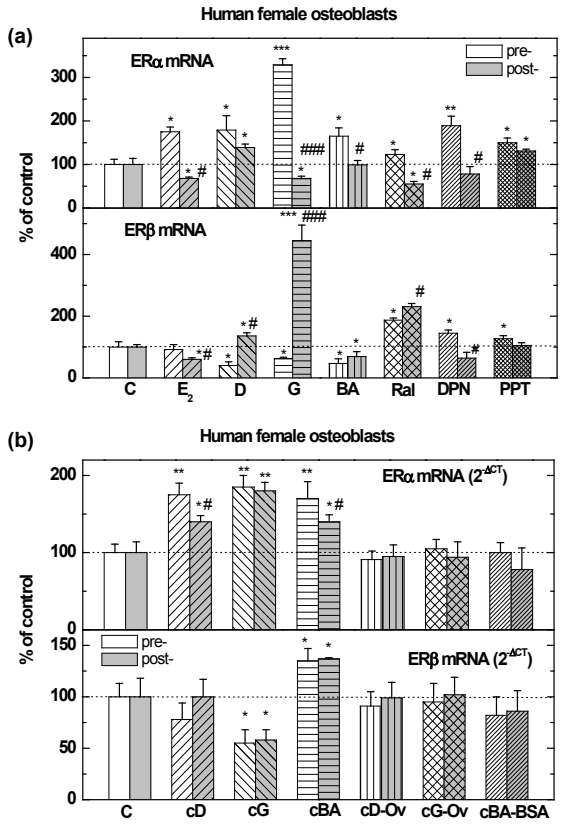

Figure 1: a. The effect of pre-treatment for 3 days of prOb and poOb with E2 (30nM), D, G, BA and Ral (3000nM) and DPN (420nM) or PPT (390nM) or **b.** carboxy derivatives (300nM) or protein-bound carboxy derivatives (3000nM) on ERα and ERβ mRNA expression. Cells were obtained, cultured, pre-treated and assayed as described. Results are means ± SEM for triplicate cultures from 5 specimen/group. Experimental means compared to control means: *, *P* <0.05; **, *P* <0.01; ***P<0.001. #P<0.05; ###p<0.001 compared with prOb.

Modulation of the expression and activity of vitamin D-1α 25 hydroxylase (1OHase) mRNA in human female cultured bone cell

Human bone-derived cells in culture derived from either pre- or post-menopausal females express vitamin D-1α 25 hydroxylase (1OHase) mRNA with higher abundance in prOb than in poOb (0.0075+0.00014 in prOb compared to 0.0041+0.00058$^{-\Delta CT}$ in poOb). The cells also produce 1, 25(OH)2D3 (1, 25) to the same extent in both Ob (60+10 in prOb compared to 75+15pg/ml in poOb). In both prOb or poOb 1OHase mRNA is modulated by different hormones (Figures 2a and 2b). In prOb 1OHase mRNA expression is stimulated only by all estrogenic compounds, whereas in cells from poOb all compounds except G and BA stimulated it but to less extent then in prOb (Figure 2a). G and BA inhibited 1OHase mRNA in poOb (Figure 2a). The cells also produce 1, 25 when incubated with 25(OH)D_3 and this production is modulated by different estrogenic compounds (Figure 2a and Figure 2b). In both Ob 1, 25 production is stimulated by all compounds except Ral but to less extent in poOb, (Figure 2a). All carboxy derivatives of the phytoestrogens stimulated both parameters but to higher extent in prOb (Figure 2b) and all the protein bound carboxy derivatives of the phytoestrogens had no effect on any of the parameters in both Ob (Figure 2b).

Modulation of expression and activity of 12 and 15 lipoxygenase (12 and 15LO) mRNA in human female cultured bone cell

Human bone-derived cells in culture derived from either pre- or post-menopausal females express 12 and 15 lipoxygenase (12LO and 15LO) mRNA with higher abundance of 12LO in prOb than in poOb (0.0403+0.0038 in prOb compared to 0.008+0.0016 $^{-\Delta CT}$ in poOb) and higher abundance of 15LO in poOb than in prOb (1.15+0.058 in prOb compared to 1.97+0.03 $^{-\Delta CT}$ in poOb). The cells also produce 12HETE and 15HETE (12H and 15H) and 12H to higher extent in prOb (1.68+0.047 in prOb compared to 1.03+0.034 pg/ml in poOb) and 15H to higher extent in poOb (1.28+0.011 in prOb compared to 1.48+0.065pg/ml in poOb).

12LO and 15LO mRNA expression was modulated by different hormones (Figure 3a and Figure 3b). In prOb and poOb 12LO mRNA expression is stimulated by all estrogenic compounds except PPT and Ral. Of note is the fact that in cells from poOb D and BA stimulated it to higher extent than in poOb (Figure 3a). In prOb and poOb 15LO mRNA expression is stimulated only by DPN and PPT but not by Ral. Of note is the fact that in cells from poOb E2, G and D stimulated it and BA stimulated it in poOb to higher extent (Figure 3a). All carboxy derivatives of the phytoestrogens did not stimulate12LO in both Ob (Figure 3b)

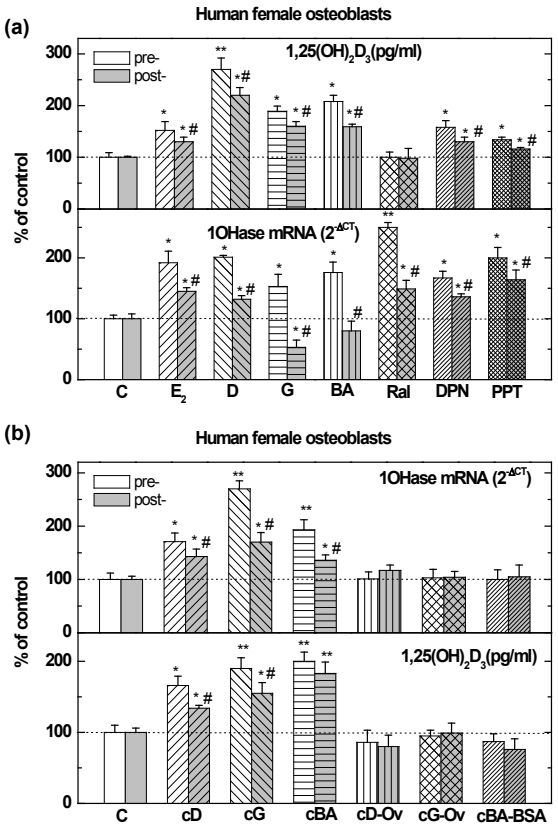

Figure 2: a. The effect of pre-treatment for 3 days of prOb and poOb with E2 (30nM), D, G, BA and Ral (3000nM) and DPN (420nM) or PPT (390nM) or **b.** carboxy derivatives (300nM) or protein-bound carboxy derivatives (3000nM) on 1OHase mRNA expression and 1,25 formation. Cells were obtained, cultured, pre-treated and assayed as described. Results are means ± SEM for triplicate cultures from 5 specimen/group. Experimental means compared to control means: *, *P* <0.05; **, *P* <0.01. #P<0.05 compared with prOb.

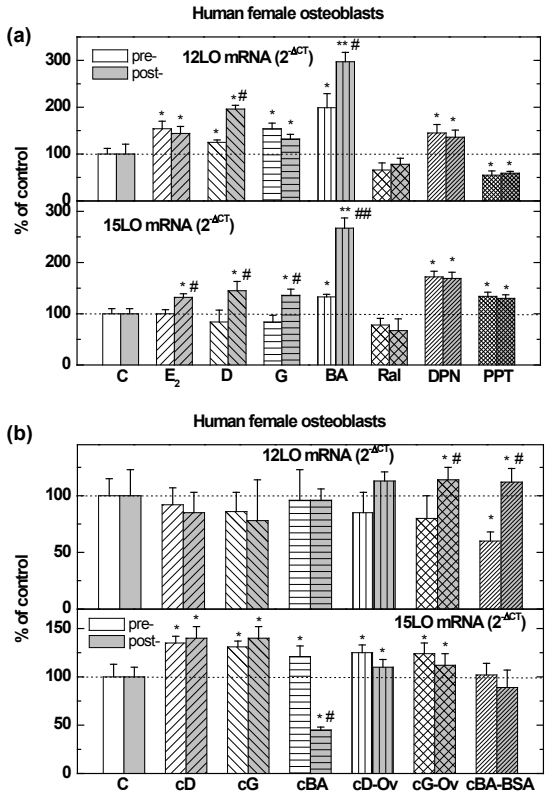

Figure 3: a. The effect of pre-treatment for 3 days of prOb and poOb with E2 (30nM), D, G, BA and Ral (3000nM) and DPN (420nM) or PPT (390nM), or **b.** carboxy derivatives (300nM) or protein-bound carboxy derivatives (3000nM) on 12LO and 15LO mRNA expression. Cells were obtained, cultured, pre-treated and assayed as described. Results are means ± SEM for triplicate cultures from 5 specimen/group. Experimental means compared to control means: *, *P* <0.05; **, *P* <0.01. #P<0.05; ##P<0.01 compared with prOb.

and all the protein bound carboxy derivatives of the phytoestrogens had slight effect on both Ob (Figure 3b). All carboxy derivatives of the phytoestrogens stimulated 15LO in both Ob but to higher extent in prOb (Figure 3b) and all the protein bound carboxy derivatives of the phytoestrogens had similar effect on both Ob (Figure 3b).

In prOb and poOb 12H formation is stimulated only by BA, DPN and PPT (Figure 4a). Of note is the fact that in poOb BA stimulated it to higher extent than in poOb (Figure 4a). In prOb and poOb 15H formation is stimulated only by BA and Ral (Figure 4a). Of note is the fact that in cells from prOb Ral stimulated it to higher extent (Figure 4a). All carboxy derivatives and their protein bound derivatives of the phytoestrogens stimulated 12H in both Ob (Figure 4b) while only cBA and cD-Ov had higher effect in prOb (Figure 4b). All carboxy derivatives of the phytoestrogens and their protein bound derivatives stimulated 15H in both Ob (Figure 4b) and only the protein bound derivatives of the phytoestrogens had higher effect in poOb (Figure 4b).

Age-dependent DNA and CK response of human female bone cells to estrogenic compounds

Human bone-derived cells in culture derived from either pre- or post- menopausal females respond to the different estrogenic compounds by stimulation of DNA synthesis (DNA) and the specific activity of creatine kinase (CK), a marker for intra cellular hormonal responsiveness (Figure 5a). In both parameters the stimulation by the different estrogenic compounds was higher in prOb except for BA (Figure 5a).

Modulation of age-dependent DNA and CK response of human female bone cells to estrogenic compounds by vitamin D less-calcemic analog

Human bone-derived cells in culture derived from either pre- or post- menopausal females respond to the different estrogenic compounds by stimulation of DNA and of CK (Figure 5a). Pre-treatment of the cells for 3 days with the less-calcemic vitamin D analog JKF (1nM/day) led to increased CK response to all estrogens except to BA (Figure 5b) and to the carboxy- derivatives of the phytoestrogens in both Ob (Figure 5c). Similar results were obtained when the changes in DNA were monitored (Figure 6a and Figure 6b).

Modulation of age-dependent intracellular and membranai E2 binding of human female bone cells to estrogenic compounds by vitamin D less-calcemic analog

Human bone-derived cells in culture derived from either pre- or post- menopausal females bind specifically estrogen to both intracellular and membranal binding sites. Pre-treatment of the cells with the less-calcemic vitamin D analog JKF (1nM/day) led to increased intracellular binding by all estrogens except to BA in both Ob (Figure 7a). On the other hand this pre- treatment led to decreased membranal binding by all estrogens in both Ob (Figure 7b).

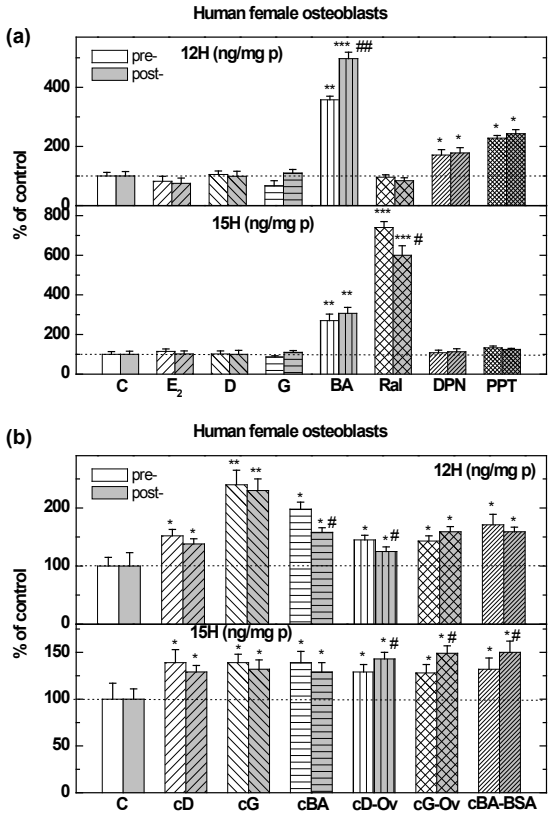

Figure 4: a. The effect of pre-treatment for 3 days of prOb and poOb with E2 (30nM), D, G, BA and Ral (3000nM) and DPN (420nM) or PPT (390nM), or **b.** carboxy derivatives (300nM) or protein-bound carboxy derivatives (3000nM) on 12HETE and 15HETE formation. Cells were obtained, cultured, pre-treated and assayed as described. Results are means ± SEM for triplicate cultures from 5 specimen/group. Experimental means compared to control means: *, *P* <0.05; **, *P* <0.01; P<0.001. #P<0.05; ##P<0.01 compared with prOb.

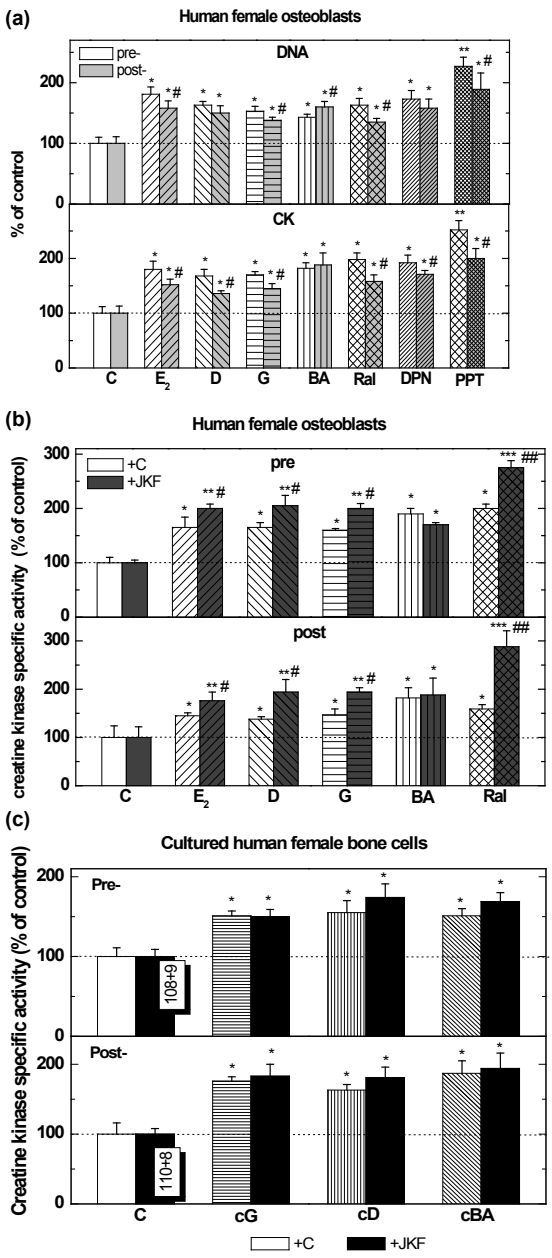

Figure 5: a. Stimulation of CK (lower panel) and DNA (upper panel) in prOb and poOb with E2 (30nM), D, G, BA and Ral (3000nM) and DPN (420nM) or PPT (390nM). Cells were obtained, cultured, treated and assayed as described. Results are means ± SEM for triplicate cultures from 5 specimen/group. Control means were 33.6±6.8 for prOb and 23.8+2.0nmol/min/mg protein for poOb for CK. Control means were 3580+215 for prOb and 2880+251 for poOb for DNA. Experimental means compared to control means: *, P <0.05; **, P <0.01. #P<0.05 compared with the level in prOb. **b.** The effect of pre-treatment for 3 days of prOb and poOb with JKF (1nM) and then treated for 24h with E2 (30nM), D, G, BA and Ral (3000nM) or **c.** carboxy derivatives (300nM). Cells were obtained, cultured, pre-treated and assayed as described. Control means were 30.6±3.8 for prOb and 22.8+1.5nmol/min/mg protein for poOb. Results are means ± SEM for triplicate cultures from 5 specimen/group. Experimental means compared to control means: *, P <0.05; **, P <0.01; P<0.001. #P<0.05; ##P<0.01 compared with prOb.

Discussion

We have previously shown age-dependent response of skeletal cells and organs to estrogenic compounds such as E2, phytoestrogens from different sources and SERMs, exemplified by raloxifene (Ral), in bone marrow and bone *in vivo* and in their derived cells in culture *in vitro*. We measured different intracellular effects such as cell proliferation determined as [^{3}H] thymidine incorporation into DNA (DNA) and energy metabolism measured by the specific activity of creatine kinase BB (CK) as well as ERs mRNA expression, 1OHase mRNA expression and activity and LO mRNA expression and activity. In the present study we decided to examine whether these results imply also to the age-dependent response of Ob derived from human derived cultured female bone cells originated from pre- or post-menopausal women (prOb and poOb respectively).

We found that the expression of ERα in Ob is higher in prOb than in poOb, whereas ERβ is the same in both Ob [18]. This might explain the higher responsiveness of "younger" Ob compared to the "older" Ob. ERα is up-regulated by all estrogenic compounds in prOb (Figure 1a) whereas in poOb all compounds except D and PPT down-regulated ERα (Figure 1a). This means that not only that the level of ERα is lower in poOb, it is down-regulated by exposure to estrogenic compounds, which might be very problematic to the bone. ERβ on the other hand was stimulated in poOb by D, G and Ral only, whereas it was inhibited in prOb (Figure 1a). If ERβ is mediating catabolic effects on bone, this might be again beneficial for the prOb. All carboxy- derivatives of the

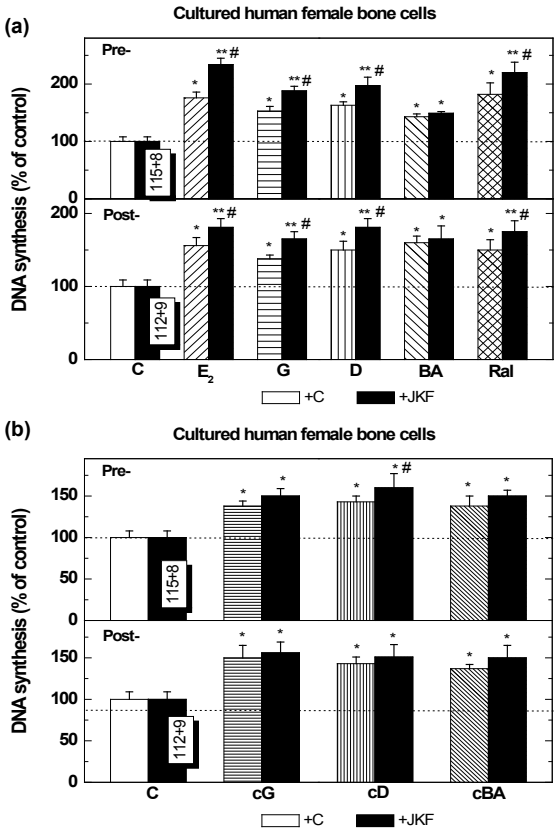

Figure 6: a. The effect of pre-treatment for 3 days of prOb and poOb with JKF (1nM) and then 24H treatment with E2 (30nM), D, G, BA and Ral (3000nM) or **b.** carboxy derivatives (300nM) for DNA. Cells were obtained, cultured, pre-treated and assayed as described. Control means were 3880+395 for prOb and 2980+281 dpm/well for poOb. Results are means ± SEM for triplicate cultures from 5 specimen/group. Experimental means compared to control means: *, P <0.05; **, P <0.01. #P<0.05 compared with prOb.

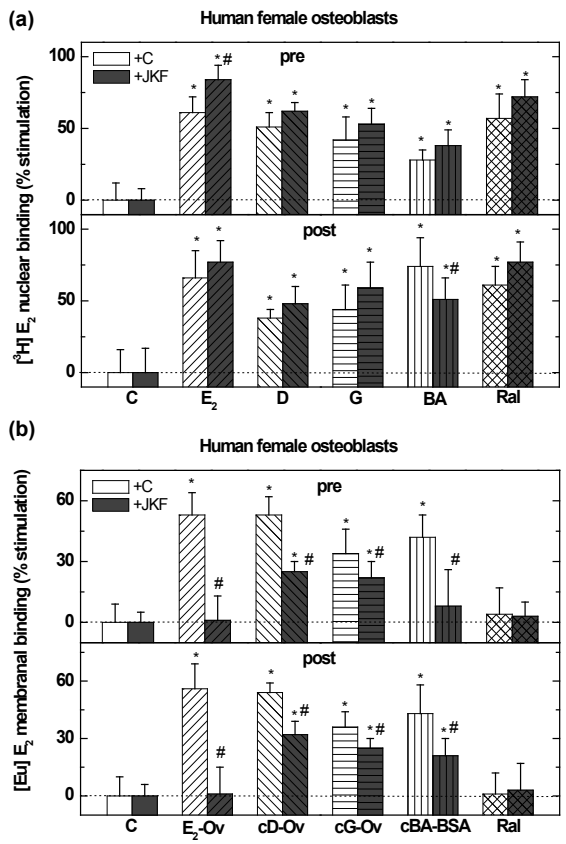

Figure 7: a. The effect of pre-treatment for 3 days of prOb and poOb with JKF (1nM) and then treated for competitive intracellular E2 binding with E2 (30nM), D, G, BA and Ral (3000nM) or **b.** E2-Ov, cG-Ov, cD-Ov and cBA-BSA (300nM) or Ral (3000nM) for membranal binding. Cells were obtained, cultured, pre-treated and assayed as described. Results are means ± SEM for triplicate cultures from 5 specimen/group. Experimental means compared to control means: *, *P* <0.05. #P<0.05 for the hormones after JKF and hormones after vehicle.

phytoestrogens, which have estrogenic activity, but not their protein-bound carboxy- derivatives of the phytoestrogens, stimulated ERα in both Ob (Figure 1b). ERβ on the other hand was stimulated in both Ob by cBA and inhibited by cG (Figure 2b). All protein- bound carboxy derivatives of the phytoestrogens had no effect on any ER in both Ob (Figure 1b). These changes in the expression of ERs might due to the fact that theprotein- bound compounds are unable to penetrate the cells and therefore not activate intra nuclear modulations. Whether these results suggest that exposing the cells to some estrogenic compounds might increase response of Ob and especially prOb is still to be examined at least in age- dependent animal models *in vivo*.

Ob also express 1OHase to higher extent in prOb than in poOb, whereas the activity of 1OHase as measured by the production of 1,25 is the same in both Ob [18]. What is the exact role of the locally produced 1, 25 in bones is not yet understood. In both age groups all estrogenic compounds including the carboxy- derivatives of phytoestrogens except Ral and the protein- bound carboxy- derivatives of phytoestrogens up-regulated 1,25 formation in prOb and in poOb but to higher extent in the younger Ob (Figure 2a, Figure 2b). Similar results were obtained when the changes in 1OHase mRNA expression was measured (Figure 2a, Figure 2b). These findings might explain some of the difference in vitamin D metabolism in the post-menopausal women which are important alone and together with estrogens for bone preservation.

We found also that Ob is expressed in both Ob. The expression of 12LO is higher in prOb than in poOb, whereas the expression of 15LO is higher in poOb than in prOb. The activity of the LO as measured by the production of 12HETE is higher in prOb than in poOb, and the production of 15 HETE is the same in both Ob [18]. In both age groups all compounds except Ral and PPT up- regulated 12LO expression, whereas E2, D, G and BA up- regulated 15LO expression only in poOb (Figure 3a). Ral did not affect 12O and 15LO in any Ob while DPN increased it in both and PPT increased 15LO and decreased 12LO (Figure 3a). All carboxy- and the protein bound- carboxy- derivatives of the phytoestrogens had small effect on both LO expression (Figure 3b). These findings might explain some of the difference in LO activity in the post-menopausal women, but in a yet unknown mechanism. In both age groups only BA, DPN and PPT up- regulated the 12LO activity resulted in increased 12H formation (Figure 4a).and only BA and Ral up- regulated the 15LO activity resulted in increased 15H formation (Figure 4a). All carboxy- and protein- bound carboxy- derivatives of the phytoestrogens up- regulated formation of both 12 and 15H in both Ob to different extent (Figure 4b). Whether this is beneficial or hazardous to the bone is not yet know.

We found that all estrogenic compounds except BA increased in both age groups Ob cell proliferation and energy metabolism (Figure 5a) but to higher extent in prOb. Again these findings indicate that "young" Ob are more active and responsive to the hormonal treatment than the "older" Ob.

The vitamin D less- calcemic analog JKF up-regulated similarly in both Ob the response of DNA and CK to the different estrogenic compounds except to BA (Figure 5b and Figure 6a). On the other hand the response to the carboxy- derivatives of the phytoestrogens was not affected by JKF (Figure 5c and Figure 6b). Ob from both age groups bind specifically E2 both to intracellular and membranal receptors similarly. JKF up- regulated competitive binding of all compounds except BA to the intracellular receptors in both Ob (Figure 7a). On the other hand JKF down- regulated competitive binding of all compounds except Ral to the membranal receptors in both Ob (Figure 7b).

Of interest is the fact that the age-dependent response of bone cells *in vivo* and *in vitro* to estrogenic compounds was modified by manipulation of the endocrine environment by vitamin D compounds to the same extent.

In conclusion cultured bone cells *in vitro* from human female pre- and post-menopausal bones respond in some of the parameters age-dependently to estrogenic by changes in intracellular and membranal parameters, in a yet unknown mechanism.

Whether or not this implies also to human bone physiology *in vivo* is yet to be established.

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