Responsiveness to estradiol-17β and to phytoestrogens in primary human osteoblasts is modulated differentially by high glucose concentration

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Abstract

We have reported previously, that female-derived bone cells responded to 17β-estradiol (E2) and to raloxifene (Ral), whereas male-derived cells responded only to dihydrotestosterone (DHT) when the stimulation of creatine kinase specific activity (CK), which is a marker for hormone responsiveness, was measured. In cells derived from pre-menopausal women, E2, G, D and Ral stimulated CK to higher extent compared to post-menopausal bone cells, whereas quercetin (Qu), carboxy-biochainin A (cBA) and carboxy-genistein (cG) stimulated CK in both age groups similarly, and biochainin A (BA) stimulated post-menopausal cells to a bit higher extent than pre-menopausal cells. Since the skeletal protective effects of estrogens are not discernable in diabetic women, we tested in this study, the effects of high glucose concentration in the growth medium, on the effects of estrogenic compounds on CK in human-derived bone cells (hObs). Female-derived hObs were grown either in normal (4.5 g/l; 22 mM, NG) or high glucose (9.0 g/l; 44 mM, HG) for 7 days. HG increased constitutive CK, but attenuated E2- and DHT-induced CK in female or male hObs, respectively. HG also inhibited genistein (G) and daidzein (D) stimulated CK in female hObs, but not the effects of biochainin A (BA), quercetin (Qu) or Ral. Intracellular, mainly nuclear binding of [3H]E2 was characteristic of the different phytoestrogens in female hObs, was abolished by HG. Membranal binding of Eu-Ov-E2, was displaced only by E2-Ov, ICI, cG-Ov or cD-Ov but decreased total binding of Eu-Ov-E2 in both age groups and completely abolished the competition with E2-Ov or ICI in both age groups, but the competition with cD-Ov and cG-Ov was decreased only slightly but not statistically significant. HG also abolished Eu-BSA-T, which bound similarly male-derived hObs. All hObs expressed mRNA for ERα and ERβ with higher abundance of ERα. HG increased mRNA for both ERs in female-derived hObs, but decreased mRNA for both ERs in male-derived hObs. Hence, human bone cells, which express specific nuclear and membranal binding sites for estrogenic compounds, are modulated by HG, leading to altered hormonal responsiveness, which might block important effects of estrogenic compounds, contributing probably to their decreased skeletal preserving properties under hyperglycemia.

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1. Introduction

We have previously studied the effects of estrogens on bone in a rat model [1–3] using the increase in the specific activity of creatine kinase as a response marker. The brain type (BB) isoenzyme of creatine kinase (CK), part of the "energy buffer" system, which regulates the cellular concentration of ATP and ADP, is the major component of
the “E2-induced protein” of rat uterus [4] and other tissues containing estrogen (E2) receptors [5]. CK stimulation is an efficient response marker to detect activity of E2, as well as other estrogenic compounds, in bone cells [6,7] particularly in osteoblasts [7], which contain low concentrations of E2 receptors [8,9]. Notably, the stimulation of CK in cultured bone cells, correlated with increased DNA synthesis in bone, requires the higher end of the physiological range of estrogen concentrations [1].

Estrogen is well known for its beneficial effect on osteoporosis [10,11]. Osteoporosis is characterized by reduction in bone mineral density, with the result of fracture after minimal trauma. The effect of estrogen in the tissues is initiated by its binding to estrogen receptors (ERs). Two ERs have been identified, ERα and ERβ, which differ in their structure and tissue distribution [12] and their biological effect [13]. Estrogen deficiency is known to be involved in osteoporosis [14], which affects every third woman above the age of 65. It has been recently reported that estrogens as well as androgens, are also necessary for the maintenance of the male skeleton [15]. Although estrogen treatment is efficient in preventing bone loss, it can also contribute to the development of estrogen-dependent tumors such as endometrial and breast tumors. Hence, new compounds, which can replace current hormone replacement therapy treatments with no such deleterious effects, are highly desirable [16].

In human-derived cultured osteoblasts (hObs), we found that E2 and DHT increased CK specific activity in a gender specific manner [17,18] as a response marker for hormonal treatment in cells containing the relevant receptors.

Phytoestrogens are a heterogeneous group of plant-derived compounds some of which are 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 than estradiol-17β [19]. Soybeans and soy foods are the most significant dietary sources of the isoflavone class of phytoestrogens, which includes genistein, daidzein and biochanin A [20] and have estrogenic action on bone and the cardiovascular system but have antiestrogenic action on the breast and the [21]. Genistein was shown to prevent bone loss in post-menopausal women [22] and in osteocarcinized rodents [23].

Diabetes has been associated with a net loss of bone [24], with reduction of formation of new bone and decrease in bone mineral density [25]. In diabetic mice the sharp up-regulation of specific transcription factors is attenuated, resulting in deficiency in conversion of mesenchymal cells to osteoblasts [25].

In the present study we analyzed the effects of high glucose on the response to gonadal steroids and to phytoestrogens of human-derived bone cells, which is relevant at least to some of the important factors existing in diabetes.

In our study we found that the response of female-derived bone cells from both age groups to some of the estrogenic compounds is blocked, probably due to decreased binding to both intracellular and membranal binding sites. Similar results were obtained with the response of male-derived bone cells to DHT. These results may lead to the use of the phytoestrogenic compounds, which are not changed by hyperglycemia, for hormone replacement therapy in diabetic patients, provided that similar results will be obtained in the relevant animal models.

2. Materials and methods

2.1. Reagents

All reagents used were analytical grade. Chemicals, 17β-estradiol (E2), dihydrotestosterone (DHT), phytoestrogens, ICI 16480 (ICI) and the creatine kinase assay kit were purchased from Sigma Chemicals Co. (St. Louis, MO).Raloxifene (Ral) was the gift of Dr. B. Fournier (Ciba-Geigy, Basel, Switzerland). The carboxy-derivatives of the phytoestrogens as previously described [26].

2.2. Cell cultures

Human bones were obtained from biopsies of patients undergoing corrective surgery following accidental injury, hip or knee replacement. All patients (women and men) were healthy, non-osteoporotic and not receiving hormonal replacement treatment. Three groups were defined: pre-menopausal women, ranging between 37 and 50 years old (n = 5 per analog treatment group, n = 10 for vehicle controls). Post-menopausal women, ranging between 59 and 84 years old (n = 5 per analog treatment group, n = 10 for vehicle controls). Men, ranging between 32 and 76 years old (n = 7 per analog treatment group, n = 10 for vehicle controls).

The non-enzymic method for isolation and culture of human bone cells and their characterization as osteoblasts was described previously [17,18]. Briefly, samples of the trabecular surface of the iliac crest or long bones were cut into 1 mm3 pieces and extensively and repeatedly washed with phosphate buffered saline (PBS) to remove blood components. The explants, with no enzymatic digestion, were seeded in 100 mm diameter tissue culture dishes and incubated in DMEM medium without CaCl2 (to avoid fibroblastic growth [17,18], containing 10% fetal calf serum (FCS) and antibiotics). Cell outgrowth from the bone explants was apparent after 6–10 days. The cultures consisted of osteoblast-like cells (with negligible fibroblasts) showing the characteristic high basal alkaline phosphatase activity and levels of ostecalcin, and their stimulated production by 1.25 dihydroxy Vitamin D3, as well as increased cAMP formation in response to PTH [17]. First passage cells were seeded at a density of 3 × 104 cells per 35 mm tissue culture dish in phenol red free DMEM with 10% charcoal stripped FCS and incubated at 37 °C in 5% CO2.

2.3. Glucose treatment

Cells were grown either in normal glucose (22 mM; NG) or high glucose (44 mM; HG) until sub-confluence for 7 days.
2.4. Hormonal treatment

At sub confluence cells were treated with 30 nM E2, 300 nM DHT, 3 μM RA, or 3 μM of each of the phytoestrogens or their carboxy-derivatives [26,27] for 24 h, followed by harvesting for CK assay.

2.5. Creatine kinase (CK) activity assay

Cells were scraped from culture dishes and homogenized by freezing and thawing three times in cold isotonic extraction buffer [17,18]. Supernatant extracts were obtained by centrifugation at 14,000 × g for 5 min at 4 °C in an Eppendorf micro-centrifuge. Creatine kinase specific activity (CK) was measured in a Kontron Model 922 Uvicron Spectrophotometer at 340 nm using a Sigma coupled assay kit (procedure 47-UV). Protein was assayed by Coomassie brilliant blue dye binding, using BSA as the standard.

2.6. Competitive binding assay for membrane impermeable, estrogenic protein conjugates

Cells cultured in 24-well plates (4 × 10³ cells/well) for 48 h were washed once with ice-cold binding medium (DMEM + 0.1% BSA and 25 mM HEPES, pH 7.4) using reaction conditions as described previously [27,28]. Subsequently, cells were incubated for 90 min at 4 °C with either of the steroid protein conjugates (estradiol-6-carboxymethyl oxime; E2-6-CMO ovalbumin, testosterone-3- (O)-carboxymethyl oxime BSA [26–29], 10 μg/ml), E2-6-CMO conjugate labeled with Europium or T-BSA conjugate control values was evaluated using a non-paired, two-tailed Student’s t-test in which n = number of donors.

3. Results

3.1. Age and sex-specific stimulation of CK specific activity by phytoestrogens in human osteoblasts

Treatment with different estrogenic compounds stimulated CK specific activity of female-derived osteoblast-like cells in diverse ways, depending on the specific compound and the sex and age of the cell donor. In cells derived from pre-menopausal women, E2, G, D and Ral stimulated CK to higher extent compared to post-menopausal bone cells (Fig. 1), whereas quecetin (Q), carboxy-biochainin A (cBA) and carboxy-genistein (cG) stimulated CK in both age groups similarly, and biochainin A (BA) stimulated post-menopausal cells to a bit higher extent than pre-menopausal cells in most of the specimen used. Both age groups did not respond to dihydrotestosterone (DHT) (Fig. 1).

When male-derived bone cells were treated with the same compounds, only DHT but none of the estrogenic compounds, stimulated CK specific activity with no age differences (data not shown).
Fig. 1. Stimulation of CK specific activity, by the different phytoestrogens: estradiol-17β (E2) or raloxifene (Ral), as well as dihydrotestosterone (DHT), in primary bone-derived cells from pre-menopausal (open bars) and post-menopausal (gray bars) women. Bone cells were cultured, treated and assayed for CK specific activity as described in Section 2. Cells were treated for 24 h with vehicle or 30 nM estradiol-17β (E2) or 3 μM of genistein (G), daidzein (D), Ral, biochanin A (BA), quercetin (Qu) or 250 nM carboxy-genistein (cG), carboxy-biochanin A (cBA) or with 300 nM DHT. Results are means ± S.E.M. for triplicate cultures from 5 women/group for each group. Control means of CK specific activity were 22.6 ± 3.2 and 20.8 ± 3.7 nmol/min/mg protein, for pre- and post-menopausal women, respectively. Experimental means compared to control means: *P < 0.05; **P < 0.01; ***P < 0.001. Means of pre-menopausal vs. post-menopausal cells: #P < 0.05.

3.2. Modulation of creatine kinase specific activity response to the different phytoestrogens by high glucose in human-derived osteoblasts

Female-derived hObs treated with E2, D and G for 24 h, showed a significant increase in CK specific activity in both age groups (Fig. 2). In all cell cultures, growth in high glucose led to abolishment of the response of CK specific activity to treatment with E2, D or G (Fig. 2) but did not abolish the response to Ral, BA or Qu in both age groups (Fig. 2). Bone cells derived from men treated with DHT showed increased CK. This response to DHT was abolished by high glucose (Fig. 3). On the other hand, CK constitutive specific activity was elevated by treatment with high glucose; in pre-menopausal cells there was 53 ± 20% increase at high glucose, in post-menopausal cells there was 129 ± 13% increase at high glucose and in male-derived cells there was 76 ± 12% increase at high glucose (Figs. 2 and 3).

3.3. Specific nuclear and membranal binding of E2 in human-derived osteoblasts

Female-derived human osteoblasts from both age groups, demonstrate intracellular which is mainly nuclear E2 specific binding as measured using [3H] E2 (Fig. 4). All estrogenic compounds tested, including the antiestrogen ICI 182780 (ICI) or the SERM raloxifene (Ral); compete for this binding in both age groups similarly. The cells also bind specifically E2-Ov-Eu to membranal binding sites, with the same profile of competition with protein-bound hormones, as well as ICI, but not with Ral or T-BSA, but with no age-dependent changes (Fig. 5). Male-derived human osteoblasts did not bind specifically Eu-Ov-E2, but bind specifically Eu-BSA/T to membranal sites (Fig. 8).
3.4. Modulation of specific nuclear binding of $^3$H$^2$E2 in human-derived osteoblasts by high glucose
Growth of female-derived osteoblasts from both age groups at high glucose concentration decreased total binding of $^3$H$^2$E2 in both age groups and abolished the specific binding by all estrogenic compounds tested (Fig. 6). Also the total binding was increased by 57–66% at both age groups.

3.5. Modulation of specific membranal binding of Eu-Ov-E2 in human-derived osteoblasts by high glucose
Growth of female-derived osteoblasts from both age groups at high glucose concentration decreased total binding of Eu-Ov-E2 in both age groups and completely abolished the competition with E2-Ov in both age groups, but the competition with cD-Ov and cG-Ov was decreased only slightly.
Fig. 8. Specific binding of different steroids to membranal binding sites in primary bone-derived cells from men grown at different glucose concentrations either normal (light gray bars) or high (dark gray bars) concentrations. Bone cells were cultured, treated and assayed for membranal binding of E2-Ov-Eu or T-BSA-Eu as described in Section 2. Results are means ± S.E.M. for percentage of specific binding of triplicate cultures in the presence of 500× fold concentration of the different hormonal-protein conjugates as competitors. Experimental means compared to control means: *P < 0.05. but not statistically significant (Fig. 7). Also the total binding was increased by 29% at both age groups.

3.6. Modulation of specific membranal binding of Eu-BSA-T in human-derived osteoblasts by high glucose

Growth of male-derived osteoblasts at high glucose concentration decreased slightly the total binding of Eu-BSA-T (Fig. 8), but abolished completely the competition with T-BSA (Fig. 8).

3.7. Expression and modulation of ERα and ERβ in human-derived osteoblasts by high glucose

Female-derived osteoblasts from both age groups as well as male-derived osteoblasts, expressed mRNA for both ERα and ERβ as measured by real time PCR (Fig. 9). The ratio of ERα to ERβ was 121 to 1 in pre-menopausal and 77 to 1 in post-menopausal and 90 to 1 in male-derived osteoblasts (Fig. 9). While the change in ERα between the female age groups was significantly lower in post-menopausal cells (P < 0.05), the changes in ERβ was not significant. High glucose increased the expression of ERα and ERβ, in female-derived cells both age groups and inhibited it in male-derived cells (Fig. 10).

4. Discussion

The phytoestrogens tested in our studies can be divided roughly into two classes on the basis of their age-dependent stimulation of CK specific activity in primary cultures of human osteoblast-like cells. Similarly to E2 and Ral (Fig. 1), the isoflavones genistein and daidzein showed higher stimulation in pre-menopausal than in post-menopausal cells. On the other hand quecertin, carboxy-biochainin A and carboxy-genistein showed similar stimulation in cells from post-menopausal and in pre-menopausal women, whereas biochainin A stimulated post-menopausal slightly more than pre-menopausal women (Fig. 1). Growing the cells in high glucose concentration (44 mM instead of 22 mM) sharpens the ability to distinguish between the two groups. First of all, the hyperglycemia increased the constitutive levels of CK by 53–129% (Fig. 2). Moreover, similarly to E2, the
stimulation of CK by genistein and daidzein was abolished by hyperglycemia in both age groups, while the effects of biochanin A and quercetin like those of Ral were not significantly changed by hyperglycemia in either age group (Fig. 2). The sex-specificity of estrogen action [1–3,18] was also demonstrated in this study in the response to phytoestrogens. Nicotiphytoestrogen tested had any effect on male-derived osteoblasts, which were stimulated only by DHT (Fig. 3). It is important to note that the constitutive levels of CK specific activity was increased by hyperglycemia also in male-derived bone cells, by 76% (Fig. 3), while the stimulation of CK by DHT in male-derived osteoblasts, was also abolished by growing the cells in high glucose concentrations (Fig. 3). In order to understand the mechanism of the changes induced by hyperglycemia in the present study, we show that the abolition of estrogenic stimulation by hyperglycemia occurs in our non-transformed human-derived primary osteoblasts, was accompanied in contrary by increases in mRNA levels of ERα and to less extent in ERβ in female cells at both ages, but with their decreases in male-derived bone cells (Fig. 10). We also analyzed total cellular (mainly nuclear) and membranarial estrogen binding to the different cells. While in normal hObs, the phytoestrogens tested, were bound to both nuclear and membranarial sites, Ral was bound only to nuclear binding sites, but they showed no age-dependent difference in the binding. This parallels our previous findings [27–29] using human vascular smooth muscle cells, where Ral showed no binding to membranarial sites and did not prevent estrogen-mediated inhibition of cell proliferation. Attempt to correlate estrogen receptors mRNAs with the changes in nuclear and/or membrane binding failed also in these vascular cells [27–29].

The effects of high glucose on abolishing nuclear binding (Fig. 5) are similar to the decreased responsiveness to phytoestrogens after growth in high glucose (Fig. 2) but opposite to the increases in ERs mRNA (Fig. 10). Also the membranarial binding of E2 but not that of some of the phytoestrogens tested was abolished by hyperglycemia (Fig. 7). Membranarial binding is therefore not correlated with the abolishment of CK specific activity stimulated by E2 and phytoestrogens in hyperglycemia. This indicates that membranarial mediated pathways are not involved in CK stimulation by estrogenic compounds tested in this study and others [28] as it is mainly nuclear receptor mediated. This finding is in accordance with our previous finding that impermeable protein-bound hormones were unable to stimulate CK in human vascular smooth muscle cells in the manner that E2 and phytoestrogen do, but they bind to membranarial binding sites [27–29].

When we assayed the changes in ERs mRNA expression by real time PCR, while ERα and ERβ mRNA were found in both ages of female-derived bone cells and in male-derived bone cells as well, hyperglycemia increased ERα and ERβ expression in female-derived cells, but decreased these mRNAs in male-derived cells. Although the biological activity and membranarial binding of the androgens in male cells are decreased by hyperglycemia, no data is available on the changes in the mRNA levels of androgen receptors.

The modulation of ERs is a recent addition to the spectrum of changes induced by hyperglycemia [27,29], which stimulates the differentiation of osteoblasts and osteoclasts and stimulates osteoblasts to produce osteocalcin and alkaline phosphatase.

Bone growth in diabetes which is disturbed [24,25] is also enhanced to the same extent by hormone replacement therapy [30] and might be the result of lower hip BMD in young women due to their type 1 diabetes [31]; therefore the use of the specific phytoestrogens and their synthetic derivatives that we use in parallel to raloxifene, might provide an alternative solution. Further studies in this direction in animal models have to be conducted for this purpose.

References