Phytoestrogenic Compounds and Their Synthetic Analogs, Contrary to Estradiol-17β Stimulates Human Derived Female Cultured Bone Cells in Hyperglycemic Conditions

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Abstract

Cultured female-derived human osteoblasts (hObs) responded by different parameters to the phytoestrogens: daidzein (D), glabrene (Gla) and glabridin (Glb), to their synthetic derivatives: carboxy-daidzein (cD) and to estradiol-17β (E1). Since the skeletal protective effects of estrogens are not discernible in diabetic women, we tested the effects of these compounds on hObs grown in growth medium with high glucose (HG; 9.0g/L; 44mM) compared to normal glucose (NG; 4.5g/L; 22mM) using the stimulation of creatine kinase specific activity (CK) and [3H]thymidine incorporation into DNA (DNA) as hormonal responsiveness markers. HG slightly increased DNA and CK in hObs. Stimulation by E1 was abolished and by cD and D was slightly decreased in HG, but not by Gla and Glb in both age groups. Growing hObs in HG upregulated the expression of mRNA of both ER and ERβ in cells from pre- but not from post-menopausal women. Cells from both age groups express also mRNA for 25 hydroxy vitamin D3, 1-α hydroxylase and showed enzymic activity which were down-regulated by HG in both age groups. Whether Gla and Glb act differentially via ERs and/or 1-α hydroxylase is not yet established. Since these compounds are active even in HG, they might be used for treating hyperglycemic/diabetic women.

Keywords: Human derived bone cells; Hyperglycemia; Estradiol-17β; Phytoestrogens; DNA synthesis; Creatine kinase

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) which is part of the ‘energy buffer’ system, regulates the cellular concentration of ATP and ADP is the major component of the "E"-induced protein of rat uterus [4] and is an efficient response marker to detect activity of E1 as well as other estrogenic compounds, in bone cells in vivo and in vitro [1,5] which contain low concentrations of E1 receptors [6,7]. 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 [1,5].

Estrogen is well known for its beneficial effect in osteoporosis [8]. Osteoporosis is characterized by reduction in bone mineral density, with the result of fracture after minimal trauma. The effect of estrogen in the different tissues is initiated by its binding to estrogen receptors (ERs). Two ERs have been identified, ER and ERβ, which differ in their structure and tissue distribution [9]. Estrogen deficiency is known to be involved in osteoporosis [10], which affects every third woman above the age of 65. Although estrogen treatment is efficient in preventing bone loss, it can also stimulate the growth of estrogen-dependent tumors. Hence, new compounds, which can replace current hormone replacement therapy treatments with no such deleterious effects, are highly desirable [11].

In human-derived cultured osteoblasts (hObs), we found that E1 increased cell proliferation and CK specific activity in a gender specific manner [12] as a response marker for hormonal treatment beyond estrogen itself in cells containing the relevant receptors.

Phytoestrogens are heterogenous 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 than E1. [13]. Soybeans and soy foods are the most significant dietary sources of the isoflavone class of phytoestrogens, which includes genistein, daidzein and biochanin A [14,15] and have estrogenic action on bone and the cardiovascular system but have anti-estrogenic action on breast cancer [16].

Diabetes has been associated with a net loss of bone [17,18], with reduction of new bone formation and decreased bone mineral density. In diabetic mice the up-regulation of specific transcription factors is attenuated, resulting in deficiency in conversion of mesenchymal cells to osteoblasts [17,18].

In the present study we analyzed the effects of high glucose on the response to phytoestrogens and their synthetic derivatives of human-derived cultured bone cells, which is relevant at least to some of the important factors existing in diabetes. The compounds we analyzed were the licoric derived compounds glabrene (Gla) and glabridin (Glb) [19], the carboxy-derivative of daidzein (cD) [20] and the phytoestrogen from soy the daidzein (D) [21] similar to the synthetic derivatives of D the DT56a (femarelle) [22].

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Received January 06, 2011; Accepted March 07, 2011; Published March 12, 2011


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Materials and Methods

Reagents

All reagents used were analytical grade. Estradiol-17β (E2), daidzein (D) and the creatine kinase (CK) assay kit were purchased from Sigma Chemicals Co. (St. Louis, MO). Carboxy-D (cD) was synthesized by us [20], licorice products: Glu and Glb were produced by us [19].

Cell cultures

Human bone cells 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 37-55 years old, (n=5). Post-menopausal women, ranging between 60-84 years old, (n=5). The non-enzymic method for isolation and culture of human bone cells and their characterization as osteoblasts was described previously [12].

Briefly, samples of the trabecular surface of the iliac crest or long bones were cut into 1mm³ pieces and extensively and repeatedly washed with phosphate buffered saline (PBS) to remove blood components. The explants, with no enzymatic digestion, were seeded in 100mm diameter tissue culture dishes and incubated in DMEM medium without Ca++. To avoid fibroblastic growth [12], containing 10% fetal calf serum (FCS) and antibiotics. Cell outgrowth from the bone explants was apparent after 6-10 days. First passage cells were seeded at a density of 3x10⁶ cells per 35mm tissue culture dish in phenol red free DMEM with 10% charcoal stripped FCS and incubated at 37°C in 5% CO₂. To obtain "high glucose" (HG) conditions, the medium including the FCS, was supplemented with glucose up to a final concentration of 44mM (9.0gm/liter). Glucose concentration in the regular medium (NG) was 22mM (4.5gm/liter).

Hormonal treatment

At sub-confluence cells were treated with 30nM E₂, 300nM cD, Glu or Glb and D at 3µM for 24h, followed by harvesting for CK or for DNA synthesis assays.

Creatine kinase (CK) extraction and assay

Cells were scraped off the culture dishes and homogenized by freezing and thawing three times in cold isotoxic extraction buffer [12]. Supernatant extracts were obtained by centrifugation at 14000xg for 5 min at 4°C in an Eppendorf micro-centrifuge.

Creatine kinase specific activity (CK) was measured in a Kontron Model 922 Uvicon Spectrophotometer at 340nm using a Sigma coupled assay kit (procedure 47-UV). Protein was assayed by Coomassie brilliant blue dye binding, using BSA as the standard [12].

Assessment of DNA synthesis

Cells were grown until sub-confluence and then treated with various hormones as indicated for CK. Twenty-two hours later [³H] thymidine was added for 2h. Cells were then treated with 10% ice-cold trichloroacetic acid (TCA) for 5min and washed twice with 5% TCA and then with cold ethanol. The cellular layer was dissolved in 0.3N NaOH, samples were aspirated and [³H] thymidine incorporation into DNA was determined [23].

Competitive binding assay for membrane estrogen binding activity

Cells were cultured in 24-well plates (3x10⁴ cells/well) for 48h and washed once with ice-cold binding medium (DMEM + 0.1% BSA and 25mM HEPES, pH 7.4) using reaction conditions as described previously [21]. Subsequently, cells were incubated for 90min at 4°C with either of the steroid protein conjugates (estradiol 6-carboxymethyl-oxime; E₂-6-CMO-ovalbumin [26-29], 10µM/well, E₂-Ov conjugate labeled with Europium (1:1000, 200µl in binding medium) were then added and the incubation was continued for another 60 minutes at 4°C. Binding was terminated by four successive washes with ice-cold binding medium. Enhancement solution (300µl/well) was then added to the cells, and the samples (200µl) were collected for fluorescence determination using an Arcus time resolved fluorometer (Wallac, Turku, Finland) [21, 23, 24]. Specific binding was defined as total binding of Europium protein conjugates minus binding in the presence of a 500 folds excess of conjugated estrogenic compounds or E₂ where appropriate.

Competitive binding assay for intracellular estrogen binding activity

Cells grown, cultured and washed as described above, were incubated for 60min at 37°C with [³H] E₂, with or without excess of different unlabelled estrogenic compounds. Binding was terminated by four successive washes with ice-cold binding medium, and cellular content of [³H] E₂ was measured in a Packard tricarb scintillation counter [21]. Specific binding was defined as the total binding of [³H] E₂ minus binding in the presence of a 500 folds excess of free estrogenic compounds.

Determination of mRNA for ERα and ER by real time PCR

RNA was extracted from cultured human bone cells, shown previously [28] to contain ERα and ERβ by western blot analysis [5], and subjected to reverse transcription as previously described [20]. For ERα, we used 5µl of cDNA in the reaction mixture with the primers 5’ AATTCGCTACAATCGAGCCCG 3’ (forward) and 5’ GTTCCTTCACACATCTCCCTCCTC 3’ (reverse). For ERβ, the same amount of cDNA was used with the primers 5’ TGGCTTGTTGGTGTTGGATGTC 3’ (forward) and 5’ TTTGCGTTTTACTGTCCTCAGC 3’ (reverse). The reaction was carried out for 30 cycles at 94°C, for 30 sec at 58°C and at 72°C for 1 min; ERα and ERβ cDNA were used as standard controls and compared to RNAase P as internal control for mRNA.

Determination of mRNA for 25 hydroxy vitamin D₃ 1-a hydroxylase (1-OHASE) by real time PCR

Total RNA from cultured hOB was extracted using the Trizol Reagent (Gibco). An aliquot of 1µg RNA from each sample was reverse transcribed (RT) using Advantage RT for PCR kit (Clontech), as previously described [25]. 1-OHase mRNA levels were analyzed using the ABI 7700 sequence detection system. Amplification of its cDNA was performed in 25µl of the sample on 96 wells plates in a reaction buffer containing Taqman universal PCR master kit. The sequences of nucleotides were as follows; forward primers: CACCCGACACGGAGCTCG; reverse primers: TCAACAGTGGACACAAACA; Taqman probe: TCGCGGCTGTGGTGCTCG. RNase P expression served as an internal control for each sample and was performed by an assay on demand gene expression products, which consists of a 20x 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 2min, 95°C for 15sec and 60°C for 1min. The total volume of the reaction was 25µl; 1.25µl universal master mix, 1.25µl 20x assay on demand mix and 11.25µl cDNA [25].
Assesment of 25 hydroxy vitamin D$_3$, 1-α hydroxylase activity

25 hydroxy vitamin D$_3$, 1-α hydroxylase activity was assessed by the measurement of 1,25 (OH)$_2$D$_3$ (1,25D) generated in hObs within 60min after the addition of 25(OH)D$_3$ (200ng/ml) to culture, using 1,25D $^{[21]}$RIA kit from DiaSorin, Mn, USA $[25]$. Protein of the layer was assayed by the Bradford method.

Statistical significance

The significance of differences between experimental and control values was evaluated using a non-paired, two-tailed Student’s $t$-test in which n=number of donors.

Results

Expression and modulation of ER$\alpha$ and ER$\beta$ in human female-derived osteoblasts by high glucose

Female- derived osteoblasts from both age groups expressed mRNA for both ER$\alpha$ and ER$\beta$ as measured by real time PCR (Figure 1). The ratio of ER$\alpha$ to ER$\beta$ was 121:1 in pre- and 77:1 in post-menopausal derived osteoblasts with no significant difference between the mRNA levels in both age groups. High glucose increased the expression of both ER$\alpha$ and ER$\beta$, in female- derived cells from both age groups with higher effect in pre-menopausal Obs (Figure 1).

Modulation of creatine kinase specific activity in response to different phytoestrogens in human female-derived osteoblasts in high glucose medium

Female derived hObs treated with E$_2$, Gla, Glb, cD or D for 24 h, showed a significant increase in CK specific activity in both age groups (Figure 2). The response of pre-menopausal cells was higher with E$_2$ and D whereas Gla was more effective in post-menopausal osteoblasts, and no age dependent difference was observed in treatments with Gla or cD (Figure 2). Growing the cells in HG increased constitutive level of the specific activity of CK in pre-menopausal osteoblasts by 46±5% and in post- menopausal osteoblasts by 34±8%. Growth of the cells in high glucose led to abolishment of the response of CK specific activity to treatment with E$_2$, slightly reduction in the response to cD or D but did not change the response to Gla or Glib in cells from both age groups (Figure 2).
Modulation of DNA synthesis response to different phytoestrogens in human female-derived osteoblasts by high glucose

Female-derived hObs treated with E₂, Gla, Glb, cD and D for 24 h, showed a significant increase in DNA synthesis in both age groups (Figure 3). The response of pre-menopausal cells was higher with E₂ and D treatments whereas no age dependent difference with the other compounds (Figure 3). Growing the cells in HG increased basal level of DNA synthesis in pre-menopausal osteoblasts by 53±20% and in post-menopausal osteoblasts by 65±13%. Growth of the cells in high glucose led to abolishment of the response of DNA synthesis to treatment with E₂, slightly reduction in the response to cD or D but did not change the response to Gla or Glb cells from both age groups (Figure 3).

Intracellular binding of the different phytoestrogens and its modulation in human female derived-osteoblasts in high glucose medium

Both pre- and post-menopausal human female-derived osteoblasts...
Modulation of specific membranal binding of the different phytoestrogens in human female-derived osteoblasts in high glucose medium

Growth of female derived osteoblasts from both age groups at high glucose concentration decreased total binding of Eu- Ov- E₁ in both age groups but did not affect the competition with E₁-BSA or C-D-Ov in both age groups, but the competition with C-D-Ov was decreased slightly but not statistically significant (Figure 5). Also the total binding was increased significantly by 29% at both age groups.

Expression and modulation of 25 hydroxy vitamin D₃, 1-α hydroxylase in human female-derived osteoblasts in high glucose medium

Female-derived bone cells from both ages expressed mRNA for 25 hydroxy vitamin D₃, 1-α hydroxylase (1-OHase) as measured by real time PCR, corrected for RNAse P mRNA (Figure 6). Growing the cells in high glucose in the medium decreased the expression of 1-OHase by about 35 to 65% respectively, in both age groups.

Modulation of 1, 25 (OH)₂D₃ production in human female-derived osteoblasts in high glucose medium

Female-derived bone cells from both age groups produced 1,25(OH)₂D₃ as measured by radio-immunoassay (Figure 6). Growing the cells in high glucose in the medium resulted decreased activity of 1-OHase measured by the production of 1,25 by about 80-70% in cells from both age groups (Figure 6).

Discussion

The estrogenic compounds tested in our studies can be divided roughly into two classes on the basis of their age dependent stimulation of DNA synthesis and CK specific activity in primary cultures of human female-derived osteoblast. Similarly to E₁, D showed higher stimulation in pre-menopausal than in post-menopausal cells (Figures 2 and 3). On the other hand C-D, Gla, Gb showed similar stimulations in cells from pre- or post-menopausal women (Figures 2 and 3). Growing the cells in high glucose concentration (44mM instead of 22mM) sharpens the ability to distinguish between the groups. First of all, the hyperglycemia increased the constitutive levels of DNA by 53-65% respectively and of CK by 46-34% (Figures 2 and 3). Moreover, the stimulation of DNA and CK by E₁ was abolished by hyperglycemia in both age groups, the stimulation of DNA and CK by C-D and D was slightly decreased by hyperglycemia in both age groups, while the effects of Gla and Gb were not significantly changed by hyperglycemia in either age group (Figures 2 and 3). It is important to note that the constitutive levels of DNA synthesis and CK specific activity were increased by hyperglycemia in age group bone cells (Figures 2 and 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 contrast, by increases in mRNA levels of ERα and to less extent in ERβ in female cells at both ages (Figure 1). We also analyzed total cellular (mainly nuclear) and membranal estrogen binding in the different cells. While in normal hObs, the phytoestrogens tested, were bound to both nuclear and membranal sites, with no age-dependent difference in the binding. This parallels our previous findings [21,23,24] using human vascular smooth muscle cells. Attempt to correlate estrogen receptors mRNAs with the changes in nuclear and/or membrane binding failed also in these vascular cells [21,23,24].

The high glucose abolished nuclear binding (Figure 4) of E₁ but not the phytoestrogenic compounds tested are parallel to the decreased responsiveness by growth in high glucose (Figures 2 and 3) but opposite to the increases in ERs mRNA (Figure 1). Also the membranal binding of E₁, as well as the other phytoestrogens tested was not affected by hyperglycemia (Figure 5). Membranal binding is therefore not correlated with the abolishment of DNA synthesis and CK specific activity stimulated by E₁ and the slight reduction of the stimulations by some of the phytoestrogens used in hyperglycemia (Figure 7a and b). This indicates that membranal mediated pathways are not involved in DNA and CK stimulation by estrogenic compounds tested in this study and others [24] 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 E₁ and phytoestrogens do, but they bind to membranal binding sites [21,23,24].

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 at higher abundance of ERα, hyperglycemia increased ERα and ERβ expression in female-derived cells (Figure 1).

The modulation of ERs is a recent addition to the spectrum of changes induced by hyperglycemia [21,23,24], which stimulates the differentiation of osteoblasts and osteoclasts and stimulates osteoblasts to produce osteocalcin and alkaline phosphatase.

Bone growth in diabetes which is disturbed [17,28] is also not enhanced to the same extent by hormone replacement therapy [26] and might be the result of lower hip BMD in young women due to their type I diabetes [27]; therefore the use of the specific phytoestrogens and their synthetic derivatives that we use in this study, might provide an alternative solution. Further studies in this direction in animal models have to be conducted for this purpose.

References


