Journal of Steroid Biochemistry & Molecular Biology 123 (2011) 25–29

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

DT56a (Femarelle), contrary to estradiol-17β, is effective in human derived female osteoblasts in hyperglycemic condition

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ARTICLE INFO

Article history:
Received 27 June 2010
Received in revised form 4 October 2010
Accepted 5 October 2010

Key words:
Human derived bone cells
Hyperglycemia
Estradiol-17β
DT56a
Femarelle
Creatine kinase

ABSTRACT

We have reported previously, that female-derived cultured osteoblasts (hObs) responded to DT56a (Femarelle) measured by the stimulation of creatine kinase specific activity (CK), which is a marker for hormone responsiveness and ^{3}[H] thymidine incorporation into DNA (DNA synthesis). Since the skeletal protective effects of estrogens are not discernable in hyperglycemic diabetic women, we sought to analyze the effect of estrogenic compounds on CK and DNA synthesis in hObs when grown in high glucose concentration (HG). Cells were grown either in normal glucose (NG) (4.5 g/L; 22 mM) or HG (9.0 g/L; 44 mM) for 7 days. HG increased constitutive CK but, the response of CK activity and DNA synthesis to estradiol-17β (E_2) treatment was reduced. In contrary, DT56a was found to be active (as measured by CK activity and DNA synthesis) in both NG and HG. HG decreases the hormonal responsiveness and might block important effects of estrogenic compounds, most likely contributing to their decreased skeletal preserving properties in hyperglycemic women. In hObs from post-menopausal women grown in HG, ERs mRNA expressions were unchanged.

On the other hand, in hObs from pre-menopausal women HG increased ERs mRNA expressions.

Since DT56a unlike E_2 is active in HG environment as well as in normal glucose, it may be an effective bone restoring agent in diabetic post-menopausal women.

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) is a part of the "energy buffer" system, which regulates the cellular concentration of ATP and ADP. CK is the major component of the "E_2-induced protein" of rat uterus [4] and other tissues containing estrogen receptors (ERs) [5]. CK stimulation is an efficient response marker to detect activity of different estrogenic compounds, in bone cells [3,6] such as osteoblasts [6,7], which contain low concentrations of estrogen receptors [8,9].

Osteoporosis is characterized by reduction in bone mineral density, with the result of fracture following minimal trauma. The beneficial effect of estrogenic compounds on osteoporosis [10,11] is initiated by the binding to ERs. Two ERs have been identified, ERα and ERβ, which differ in their structure, tissue distribution [12] and biological activity [13]. Estrogen deficiency is known to be involved in osteoporosis [14], which affects every third woman above the age of 65 [14,15].

In human female-derived cultured osteoblasts (hObs), we found that E_2 increased CK and DNA synthesis in a gender specific manner [16,17]. As a search for a more selective treatment for menopause, we looked at different botanical compounds to modulate ERs [18–20].

DT56a (Femarelle®, Se-cure Pharmaceuticals, Dalton, Israel) is a unique standardized botanical compound that was seen to act as a SERM. Femarelle increased bone mineral density in post-menopausal women [21] and relieved vasomotor symptoms with no effect on sex hormone levels or endometrial thickness [21]. Its SERM properties were shown in previous studies in rat model [22,23] and in MCF-7 human breast cancer cell-line [24]. DT56a also stimulated bone formation in a rat model, shown by histological and histomorphometrical parameters [25]. DT56a stimulated CK and DNA synthesis as well as other parameters [23] in both pre- and post-menopausal female bone cells with maximal effect at 100 ng/ml for both age groups.

Diabetes has been associated with a net loss of bone [26], with reduction of formation of new bone and decrease in bone mineral density [27]. In diabetic mice the up-regulation of specific transcription factors is attenuated, resulting in deficiency in conversion of mesenchymal to osteoblasts cells [27]. Diabetic women are at higher risk for osteoporotic fractures [28–32], thus the challenge is to find an agent that will be active also in high glucose milieu.

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doi:10.1016/j.jsbmb.2010.10.001

In the present study we analyzed the effects of high glucose in the growth medium of hObs on the response to DT56a as compared to E_2. In a previous study [34] we found that the response of hObs (derived from both pre-menopausal and post-menopausal women) to E_2 was blocked in HG, probably due to decreased binding to both intracellular and membranal binding sites. In the current study we compared the effect of DT56a, to E_2 on hObs grown in high glucose (HG), in both age groups.

2. Materials and methods

2.1. Reagents

All reagents used were of analytical grade. Chemicals, estradiol-17β (E_2), and creatine kinase (CK) assay kit were purchased from Sigma Chemicals Co. (St. Louis, MO). DT56a was provided by Se-cure Pharmaceuticals (Dalton, Israel).

2.2. Cell cultures

Human female osteoblasts were obtained from biopsies of patients undergoing corrective surgery following accidental injury, hip or knee replacement. All patients were healthy, non-osteoporotic and not receiving hormone therapy (HT). Two groups were defined: pre-menopausal women, ranging between 37 and 55 years old ($n = 5$) and post-menopausal women, ranging between 60 and 84 years old ($n = 5$). The non-enzymatic method for isolation and culture of human bone cells and their characterization as osteoblasts was described previously [16–17]. In brief: samples of the trabecular surface of the iliac crest or long bones were cut into 1 mm^3 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 Ca^{++} (to avoid fibroblastic growth) and 10% fetal calf serum (FCS) and antibiotics. Cell outgrowth from bone explants was apparent after 6–10 days. The cultures consisted of osteoblast-like cells (with no fibroblasts) showing the characteristic of osteoblasts: high alkaline phosphatase (ALP) activity, dose-dependent increase of ALP by 1,25-$(OH)_2D_3$, high levels of parathyroid hormone (PTH)-induced cyclic AMP, and 1,25-$(OH)_2D_3$-induced osteocalcin [16,17]. First passage cells were seeded at a density of 3×10^5 cells per 35 mm tissue culture dish in phenol red free DMEM with 10% charcoal stripped FCS and incubated at 37 °C in 5% CO_2. To obtain "high glucose" (HG) conditions, the medium including the FCS, was supplemented with glucose up to a final concentration of 44 mM (9 g/L). Glucose concentration in the regular medium (NG) was 22 mM (4.5 g/L) [34].

2.3. Treatment of human osteoblasts

At sub-confluence cells were treated with 30 nM of E_2 or 200 ng/ml DT56a [23] for 24 h, followed by harvesting for CK assay or for DNA synthesis.

2.4. DNA synthesis in human osteoblasts

Cells were grown until sub-confluence and treated with various compounds as indicated. Twenty-two hours following the treatment, [^{3}H] thymidine was added for 2 h. Cells were then treated with 10% ice-cold trichloroacetic acid (TCA) for 5 min and washed twice with 5% TCA and 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 [23].

2.5. Creatine kinase (CK) activity assay in human osteoblasts

Cells were grown until sub-confluence and treated with various compounds as indicated. Twenty four hours following the treatment, cells were scraped from culture dishes and homogenized by freezing and thawing three times in cold isotonic extraction buffer [16–17]. Supernatant extracts were obtained by centrifugation at $14{,}000 \times g$ for 5 min at 4 °C in an Eppendorf micro centrifuge. CK was measured in a Kontron Model 922 Uvicon 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 [23].

2.6. Expression of ERα and ERβ by real time PCR in human osteoblasts

RNA was extracted from cultured osteoblasts and subjected to reverse transcription as previously described [34,37]. 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 30 cycles at 94 °C for 30 s, at 57 °C for 30 s and at 72 °C for 1 min. For ERβ, the same amount of cDNA was used with the primers 5′ TGCTTTGGTTTGGGTGATTGC 3′ (forward) and 5′ TTTGCTTTTACTGTCCTCTGC 3′ (reverse) for 30 cycles at 94 °C for 30 s, at 58 °C for 30 s and at 72 °C for 1 min. cDNAs for ERα and ERβ were used as standard.

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

Cells 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 [34,37].

2.8. Statistical analysis

Results are given as mean ± SEM. Statistical analysis for the significance of differences between experimental and control values was evaluated using a non-paired, two-tailed Student's *t*-test in which *n* is the number of donors.

3. Results

3.1. Modulation of DNA synthesis response to DT56a and E_2 by high glucose in human female-derived osteoblasts

Female derived hObs treated with 30 nM E_2 and 200 ng/ml DT56a for 24 h, showed a significant increase in DNA synthesis in both age groups (Fig. 1). Growth in HG led to diminished response of cell proliferation to treatment with E_2 but had no effect on the response to DT56a in both age groups (Fig. 1).

3.2. Modulation of creatine kinase specific activity response to DT56a and E_2 by high glucose in human female-derived osteoblasts

Female derived hObs treated with 30 nM E_2 and 200 ng/ml DT56a for 24 h, showed a significant increase in CK specific activity in both age groups (Fig. 2). Growth in HG led to diminished response of CK to treatment with E_2 but did not change the response to DT56a in both age groups (Fig. 2). In addition, CK constitutive specific activity was elevated by treatment with HG; in pre-menopausal cells there was a 53 ± 20% increase at high glucose, and in post-

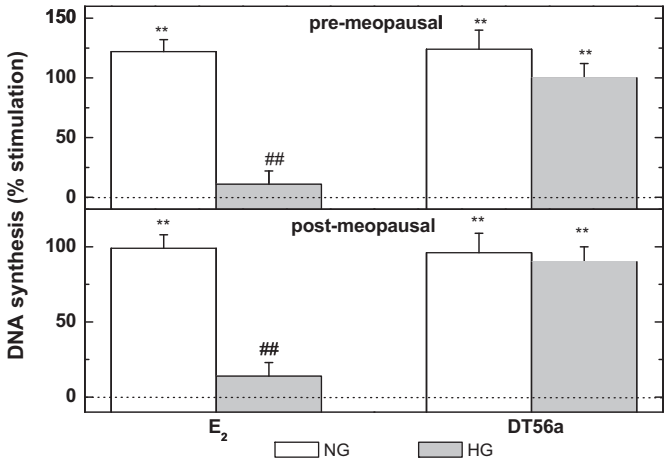

Fig. 1. Stimulation of ^{3}[H] thymidine incorporation into DNA synthesis, by estradiol-17β or DT56a, in primary bone-derived cells. Cells from pre- (upper panel) and post-menopausal (lower panel) women were grown in NG medium (white bars) or in HG medium (grey bars). Bone cells were cultured, treated and assayed for DNA synthesis as described in Section 2. Cells were treated for 24 h with control vehicle, 30 nM E_2 or 200 ng/ml DT56a. Results are means ± SEM for triplicate cultures, from 5 women/group for each age group. Control means (at NG) of DNA synthesis were 6220 ± 302 and 5908 ± 337 dpm/well, for cells from pre- and post-menopausal women respectively. Experimental means compared to control means: $^{**}P < 0.01$; HG means compared to NG means: $^{\#\#}P < 0.01$.

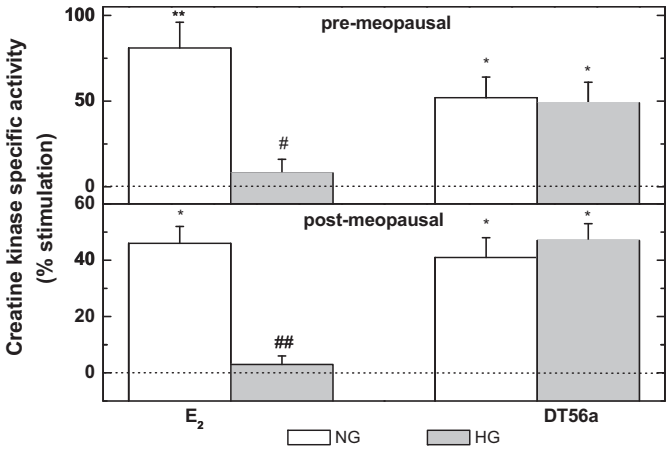

Fig. 2. Stimulation of creatine kinase specific activity, by estradiol-17β or DT56a, in primary bone-derived cells. Cells from pre- (upper panel) and post-menopausal (lower panel) women are grown in NG medium (white bars) or HG medium (grey bars). Bone cells were cultured, treated and assayed for CK as described in Section 2. Cells were treated for 24 h with vehicle, 30 nM E_2 or 200 ng/ml DT56a. Results are means ± SEM for triplicate cultures from 5 women/group for each age group. Control means (at NG) of CK 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: $^{*}P < 0.05$; $^{**}P < 0.01$; means of HG vs NG $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

menopausal cells there was a 129 ± 13% increase at HG (data not shown).

3.3. Modulation of the expression of mRNA of ERα and ERβ by high glucose in human female-derived osteoblasts

The expression of mRNA for ERα and ERβ was determined in hObs by real time PCR. RNA was extracted from cells, and was subjected to reverse transcription as described in Section 2. Both age group-derived cells express mRNA for both ERα and ERβ with a ratio of ERα to ERβ of 98:1.0 in pre-menopausal cells and 231:1 in post-menopausal cells (Fig. 3). When cells were grown in HG (8 extracts), there was no change in the expression of mRNA for ERα or

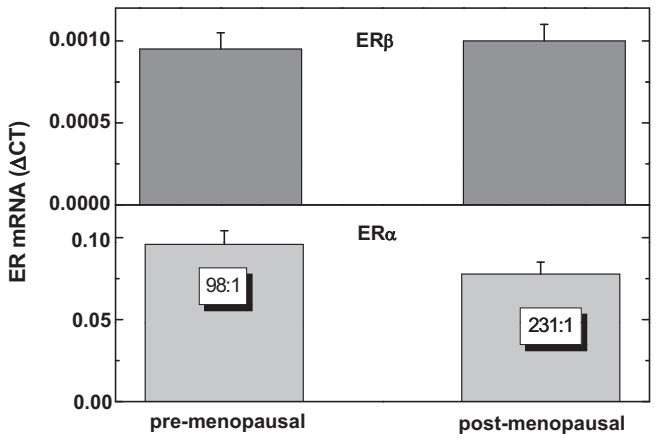

Fig. 3. Real time PCR assay of mRNA levels of ERα and ERβ in primary bone-derived cells from pre- and post-menopausal women. Cells were grown and assayed as described in Section 2. ERβ (upper panel) and ERα (lower panel) in cells from pre-menopausal women (white bars) or post-menopausal women (grey bars) were determined and results are means of 5 experiments and are expressed as number of cycles needed to get these mRNA levels (ΔCT).

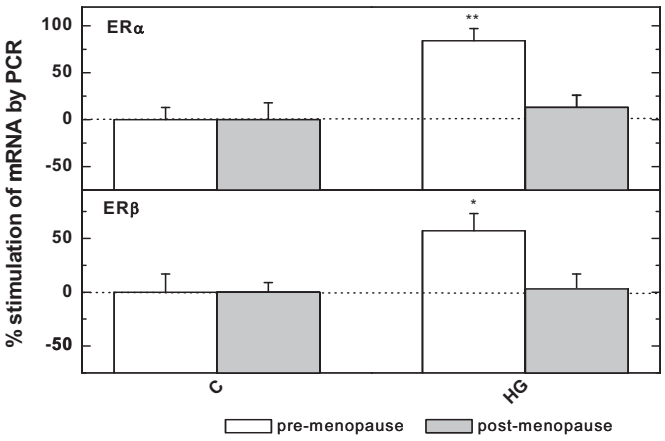

Fig. 4. The effect of growth in HG medium on mRNA levels of ERα and ERβ in primary bone-derived cells. ERα (upper panel) and ERβ (lower panel) cells from pre- (white bars) and post-menopausal (grey bars) women were measured by real time PCR. Results are means of 4 experiments and are expressed as % change in mRNA levels compared to cells growing in normal glucose: $^{*}P < 0.05$; $^{**}P < 0.01$.

ERβ in post-menopausal cells while in pre-menopausal cells there was an increase of 50–60% in the expression of mRNA for both ERs (Fig. 4).

3.4. Modulation of intracellular binding of DT56a to ER receptors by high glucose in human female-derived osteoblasts

Female derived hObs cells demonstrated E_2 specific binding of ^{3}[H] E_2 (data not shown), presumably predominantly nuclear under these conditions (37 °C for 60 min). E_2 and DT56a competed with ^{3}[H] E_2, showing significant specific binding (Fig. 5). Growing the cells in HG resulted in no change in the intracellular specific estrogen binding of DT56a but diminished binding of E_2 (Fig. 5). The basal binding of ^{3}[H] E_2 was decreased in HG by 37% in pre-menopausal cells (lower panel) and by 66% (upper panel) in post-menopausal cells (Fig. 5).

4. Discussion

Starting from the middle of the 20th century, Diabetes Mellitus became one the biggest epidemics of the western world. 10.7%

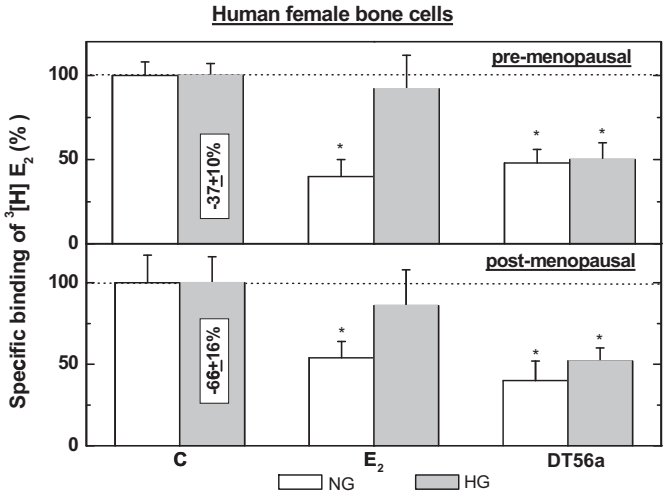

Fig. 5. The effect of growth in HG medium on intracellular binding of E_2 and DT56a in primary bone-derived cells. Cells from pre- (lower panel) and post-menopausal (upper panel) women, were monitored for binding of E_2 and DT56a as measured by competition of the binding of ^{3}[H] E_2. Results are means of 4 experiments and are expressed as % modulation of the binding in HG cells (grey bars) compared to cells growing in normal glucose (NG, white bars): $^*P < 0.05$.

of people over the age of 20 have diabetes. 23.1% of people over the age of 60 have diabetes and 11.5 million women in the United States aged 20 or older have diabetes [35]. In the year 2000, there were approximately 171 million people with diabetes; estimates for 2030 suggest that the prevalence of this disease will increase to 366 million, most of which will be type 2 diabetes [35]. Hormonal therapy for the management of menopause was shown to be less effective in diabetic women [26–33]. Thus, there is an immediate need for a treatment that will be effective in diabetic menopausal women. In the current study we compared the effect of a novel SERM-DT56a to E_2 on hObs.

E_2 showed lower stimulation in post-menopausal than in pre-menopausal cells in both NG and HG. On contrary, DT56a showed similar stimulation of hObs derived both from pre-menopausal and post-menopausal women (Figs. 1 and 2). Growing the cells in HG (44 mM vs 22 mM glucose) sharpens the ability to distinguish between E_2 and DT56a. First, HG increased the constitutive levels of CK by 53–129% with no significant change in basal DNA synthesis. Moreover, the stimulation of cell proliferation and CK was diminished by hyperglycemia in both age groups by E_2, whereas the effects of DT56a on both CK and DNA synthesis were not significantly altered by hyperglycemia in either age group (Figs. 1 and 2). In order to try to understand the mechanism of the changes induced by hyperglycemia, we analyzed whether the reduction of estrogenic stimulation by hyperglycemia occurred in our non-transformed human-derived primary osteoblasts, and whether it correlated with changes in the expression of mRNA for the ERs. We found that hyperglycemia was accompanied by an increase in mRNA levels of ERα and to a lesser extent in ERβ in pre-menopausal women hObs, but with no change in post-menopausal bone cells.

High glucose diminished nuclear binding to ERs [34]. This can explain the decreased responsiveness to E_2. Membranal binding of some plant-based compounds, were found not to be affected by hyperglycemia [34]. This may explain the unchanged response to DT56a in HG, suggesting that its effects might be mediated via membranal binding sites.

The modulation of ERs is a recent addition to the spectrum of changes induced by hyperglycemia [34,37], which stimulates the differentiation of osteoblasts and osteoclasts and stimulates osteoblasts to produce osteocalcin and alkaline phosphatase.

Bone growth in diabetes was found to be disturbed [26,27] and less responsive to hormone replacement therapy [32,33] resulting in lower hip BMD and higher prevalence of fractures [28–33].

The key finding in the present study is a clear distinction between the effect of estradiol-17β and DT56a on human derived osteoblasts. While the effect of estradiol-17β was significantly affected in HG environment, the effect of DT56a was not. These results may indicate towards the benefit of use of DT56a in diabetic women.

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