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High Glucose Modulates Responsiveness to Estrogens of Human Derived Female Cultured Osteoblasts and in Osteoblastic Cell Lines

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Abstract

Human female- derived osteoblast- like cells in culture (hObs) and the cell lines (SaOS2 and hFOB) express mRNAs involved in bone biology and physiology such as estrogen receptor α (ERα) and α (ERα), vitamin D receptor (VDR), 1α, 25 (OH) vitamin D_3 hydroxylase (1OHase) and 12 and 15 lipoxygenases (12LO and 15LO). These mRNAs are modulated by estrogenic compounds. Since the skeletal protective effects of estrogens are not discernible in diabetic women, we tested the estrogenic modulations of these parameters in cells grown in growth medium containing high glucose (HG; 9.0g/L; 44mM) compared to normal glucose (NG; 4.5g/L; 22mM). HG significantly increased DNA synthesis (DNA) and creatine kinase specific activity (CK). Stimulations of DNA but not of CK by estradiol-17β (E_2), by 4, 4', 4"-[4-propyl-(1H)-pyrazol-1, 3, 5- triyl] tris-phenol (PPT; ERa specific agonist), or by 2, 3-bis (4-hydroxyphenyl)-propionitrile (DPN; ER β specific agonist), were modulated by HG. HG Itself up regulated the expression of mRNA of 12LO and 15LO and to less extent ERβ and VDR, but had no effect on ERα and 1OHase mRNA expression. The different hormonal treatments modulated the expressions of VDR, 1OHase, 12LO and 15LO mRNAs which were reduced in HG, whereas the induction of their products 1α, 25 dihydroxy-vitamin D_3 (1,25D) and 12- and 15- hydroxyeicosatetraenoic acid (12 and 15 HETE) were only slightly affected by HG. The exact mechanism of HG effects on bone cell responses and its relationship to human bone physiology is not yet clear.

Keywords: Human Osteoblasts; Hyperglycemia; ERs Specific Analogs; ERs; VDR; 1OHase; Los; HETEs; 125D; Estrogens

Introduction

We have previously studied the effects of estroges on rat bone physiology (Somjen et al 1989; Kaye *et al* 1990) using the increase of the specific activity of creatine kinase (CK) as a response marker [1,2]. The brain type (BB) iso-enzyme of CK which is part of the "energy buffer" system, regulates the cellular concentration of ATP and ADP, and is a response marker to different hormones including estrogenic compounds, in bone cells *in vivo* and *in vitro* (Reiss and Kaye 1981; Fournier *et al* 1996), which contains detectable concentrations of E2 receptors (Komm *et al* 1988; Enmark and Gustafsson 1999) [3-6].

Estrogens are known for their beneficial effect in osteoporosis (Komm *et al* 2002), characterized by reduction in bone mineral density, leading to fractures after minimal trauma. The biological effect of estrogens in cells and 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 (Enmark and Gustafsson 1999) [6]. 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 the current used hormonal therapy treatments with no such deleterious effects, are highly desired (Delmas 2000) [7].

In human- derived cultured osteoblasts (hObs), we found that estradiol 17β (E_2) increased cell proliferation and CK specific activity in a gender specific manner (Katzburg *et al* 2001) [8].

Diabetes is associated with a net loss of bone (He *et al* 2004 and Consensus opinion of The North American Menopause Society 2000), 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 (He *et al* 2004 and Consensus opinion of The North American Menopause Society 2000) [9,10].

We have found previously that E_2 and other estrogenic compounds stimulate also vitamin D receptor (VDR) and 1OHase mRNA expression and activity, measured by 1,25D formation (Somjen *et al* 2008;2010;2011), as well as lipoxygenase mRNA (12 and 15LO) expression and activity, measured by HETE formation (12 and 15HETE) (Somjen *et al* 2007; 2011) [11,12].

We now analyzed the effects of high glucose (HG) compared to normal glucose (NG) in the growth medium on the response to estrogenic compounds of human-derived cultured bone cells, as well as the human female bone cell lines the $SaSO_2$ and hfoB, which is relevant to the important factors existing in diabetes. The compounds analyzed were E_2 and the ERα and ERβ specific analogs i.e. DPN and PPT.

In the present study we analyzed:

1. Modulation by NG and HG of DNA and CK basal activities and their responses to treatment with E_2, DPN and PPT.
2. Modulation by NG and HG of ERs basal expression and their responses to treatment with E_2, DPN and PPT.
3. Modulation by NG and HG of VDR and 1OHase expression as well as 1,25D formation and their responses to treatment with E_2, DPN and PPT.
4. Modulation by NG and HG of 12 and 15 LO expression as well as 12 and 15HETE formation and their responses to treatment with E_2, DPN and PPT.

Materials and Methods

Reagents

All reagents used were of analytical grade. E_2, DPN, PPT and creatine kinase (CK) assay kit were purchased from Sigma Chemicals Co. (St. Louis, MO).

Cell Cultures

1. 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- 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 $3x10^5$ cells per 35mm 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 44nM (9.0gm/liter). Glucose concentration in the regular medium (NG) was 22nM (4.5gm/liter).
2. $SaSO_2$ and hfoB human female derived cell lines were obtained from ATCC and were grown as previously done.

Hormonal treatment

At sub-confluence cells were treated with 30nM E_2, 300nM DPN or PPT at 3μM for 24h, followed by harvesting for the different assays.

Creatine kinase (CK) Extraction and Assay

Cells were scraped off the culture dishes and homogenized by freezing and thawing three times in cold isotonic extraction buffer. 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 (Kaye *et al* 1990) [2].

Assessment of DNA Synthesis

Cells were grown until sub- confluence and then treated with various hormones as indicated for CK. Twenty-two hours later [^{3}H] 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 0.3ml of 0.3N NaOH, samples were aspirated and [^{3}H] thymidine incorporation into DNA was assayed (Somjen *et al* 1989) [1].

Determination of mRNA for ERa , ERb, VDR and 25 hydroxy vitamin D3 1-α hydroxylase (1OHase), 12LO and 15LO by real time PCR

RNA was extracted from cultured human bone cells, and subjected to reverse transcription as previously describedfor the different parameters (Somjen *et al* 2005,2007,2010a,2011b) [13-15].

Assesment of 25 Hydroxy Vitamin D_3 1-a Hydroxylase Activity

1OHase activity was assessed by the measurement of 1,25 $(OH)_2D_3$ (1,25D) generated in hObs within 60min after the addition of 25(OH)D3 (200ng/ml) to culture, using 1,25D 125I RIA kit from DiaSorin, Mn, USA (Somjen et al 2012). Protein was assayed by Coomassie brilliand blue dye binding, using BSA as standard.

Assessment of 12 and 15LO Activity

12 Lo and 15Lo activities were assessed by measuring 12HETE and 15HETE Formation. Cells and medium were extracted for HETE formation and analyzed by HPLC as previously described (Somjen et al 2011a) [16].

Statistical Significance

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

Results

Modulation of DNA Synthesis and CK Specific Activity by E_2 and ERs Specific Agonists in Human Female-Derived Osteoblasts by HG

Basal activities of the different cells show that hObs from pre-menopausal females show the highest activity of both parameters (Figure 1a). Growing the cells in HG increased constitutive level of the specific activity of CK in pre-menopausal osteoblasts by 146+5 % and in post- menopausal osteoblasts by 134+8 %, in SaOS2 by 135+15 % and no effect in hfoB 110+8 % (Figure 1b).

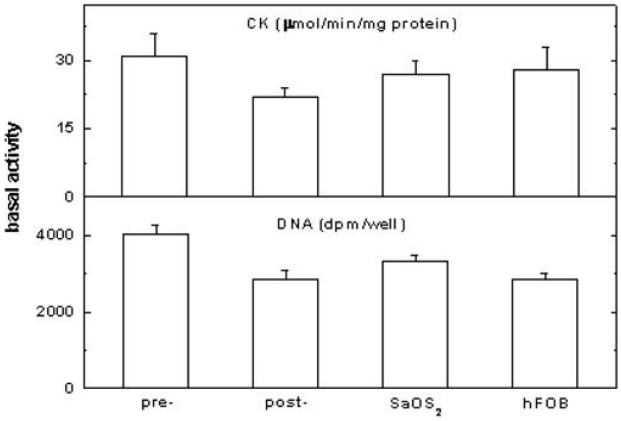

Figure 1a: Basal levels of DNA synthesis and CK specific activities in primary human female- derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for analysis as described in Materials and Methods. Results are means +SEM for triplicate cultures from 5 donors for each group

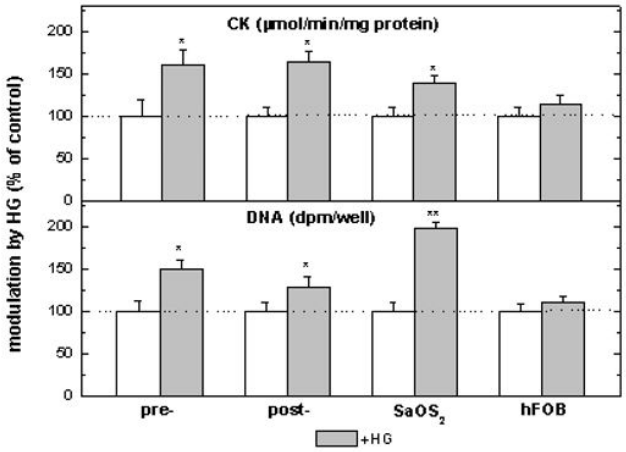

Figure 1b: Modulation by HG (44nM compared to 22nM)) of basal levels of DNA synthesis and CK specific activities in primary human female- derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for analysis as described in Materials and Methods. Results are means +SEM for triplicate cultures from 5 donors for each group

Growing cells in HG increased basal level of DNA synthesis in pre-menopausal osteoblasts by 153+20 % and in post- menopausal osteoblasts by 165+13%, in $SaOS_2$ by 200+12 % but not in hfoB 108+8 % (Figure 1b). Female derived hObs treated with E_2, DPN or PPT for 24h, showed a significant increase in CK, in both age groups and both cell lines (Figure 2b). The response of pre-menopausal cells was higher than post- and more than $SaOS_2$ and the same as in hfoB with E_2 (Figure 2b).

Growth of the cells in HG led to reduction of the response of CK to treatment with E_2, DPN and PPT in cells from both age groups and in both cell lines (Figure 2a). Growth of the cells in HG led to abolishment of the response of DNA to treatment with E_2 or DPN but not to PPT in cells from both age groups and in both cell lines (Figure 2a).

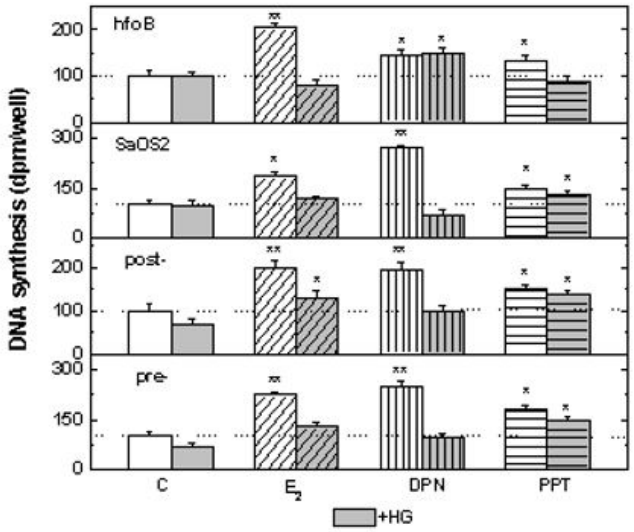

Figure 2a: Modulation by HG (44nM compared to 22nM) of the stimulation by E_2, DPN and PPT of DNA synthesis in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained, cultured, treated, and extracts prepared for analysis as described in Materials and Methods. Results are means ±SEM for triplicate cultures from 5 donors for each group. Means of cells grown in high glucose (grey bars) were compared to cells grown in normal glucose (open bars): *, P <0.05; **, P <0.01.

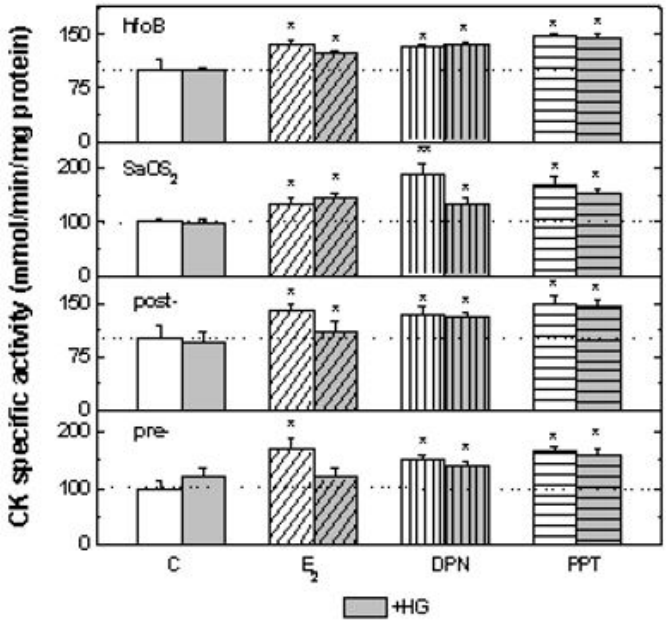

Figure 2b: Modulation by HG (44nM compared to 22nM) of the stimulation by E_2, DPN and PPT of CK specific activity in primary human female- derived osteoblasts and SaOS2 as well as hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for analysis as described in Materials and Methods. Results are means ±SEM for triplicate cultures from 5 donors for each group. Means of cells grown in high glucose (grey bars) were compared to cells grown in normal glucose (open bars): *, P <0.05; **, P <0.01

Expression and Modulation of Erα and Erβ in Human Female-Derived Osteoblasts by HG

Female- derived osteoblasts from both age groups and both cell lines expressed mRNA for both ERα and ERβ as measured by real time PCR (Figure 3a). High glucose modulated the expression of both ERα and ERβ, in these cells to different extents (Figure 3b).

Female derived hObs treated with E_2, DPN or PPT showed a significant modulations in ERα, in all cells with different patterns of modulations of these effects by HG (Figure 4a and b). Similar results were obtained when ERβ was assayed (Figure 4b).

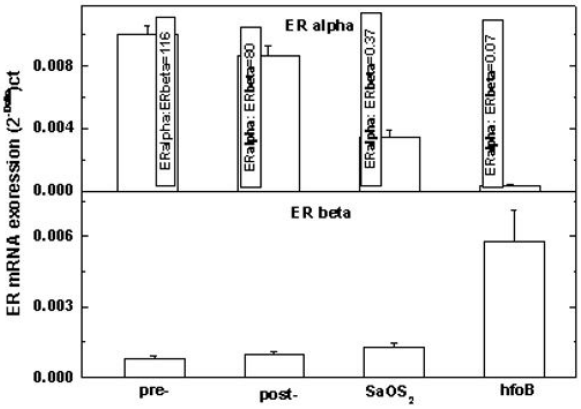

Figure 3a: Basal expression of ERα and ERβ mRNAs in primary human female354 derived osteoblasts as well as $SaOS_2$ and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for analysis asdescribed in Materials and Methods. Results are means ±SEM for triplicate cultures from 5 donors for each group

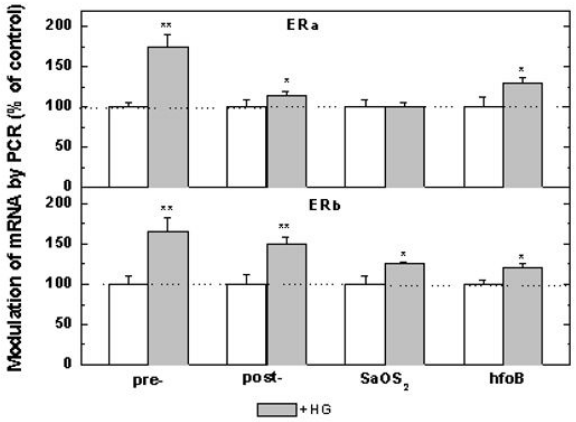

Figure 3b: Modulation by HG (44nM compared to22nM)) on basal expression of ERα and ERβ mRNAs in primary human female derived osteoblasts as well as $SaOS_2$ and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for analysis as described in Materials 361 and Methods. Results are means ±SEM for triplicate cultures from 5 donors for each group.

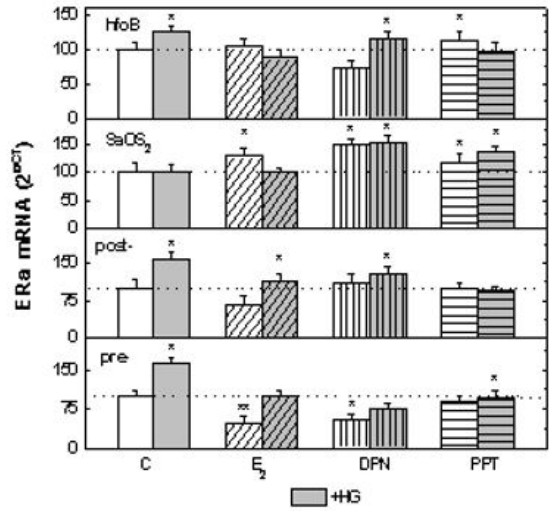

Figure 4a: Modulation by HG (44nM compared to 22nM) of the modulation by E_2, DPN and PPT of the expression of mRNA for ERα in primary human female derived osteoblasts and SaOS2 as well as hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for real time PCR 367 analysis as described in Materials and Methods. Results are means ±SEM for triplicate cultures from 5 donors for each group. Means of cells grown in high glucose (grey bars) were compared to cells grown in normal glucose (openbars): *, P <0.05; **, P <0.01.

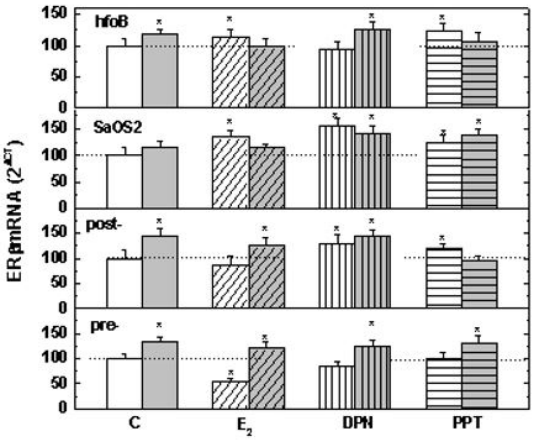

Figure 4b: Modulation by HG (44nM compared to 22nM)) of the modulation by E_2, DPN and PPT of the expression of mRNA for ERβ in primary human female- derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for real time PCR analysis as described in Materials and Methods. Results are means ±SEM for triplicate cultures from 5 donors for each group. Means of cells grown in high glucose (grey bars) were compared to cells grown in normal glucose (open bars): *, P <0.05; **, P <0.01.

Expression and Modulation of VDR and 1OHase in Human Female-Derived Osteoblasts by HG

Female-derived bone cells from both age groups and cell lines expressed mRNA for VDR and 1OHase as measured by real time PCR, corrected for RNAse P mRNA and also produced 1,25D as measured by radio immune assay (Figure 5a). Growing the cells in HG decreased the expression of 1OHase as well as 1,25D production, in both age groups and both cell lines (Figure 5b). While growing the cells in HG increased in all cells tested, Cells treated with E2, DPN or PPT, showed a significant modulation of VDR (Figure 5b and 6a). In all cells HG increased its expression (Figure 6a). Similar results were obtained when 1OHase was assayed for both mRNA expression and 1,25D production (figure 6b and c).

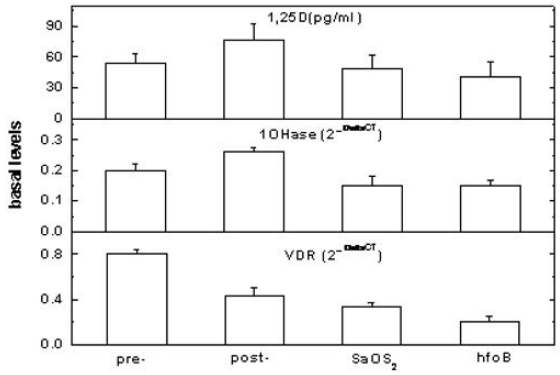

Figure 5a: Basal expression of VDR and 1OHase mRNAs and 1,25D formation in primary human female- derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for analysis as described in Materials and Methods. Results are means ±*SEM for triplicate cultures from 5 donors for each group*

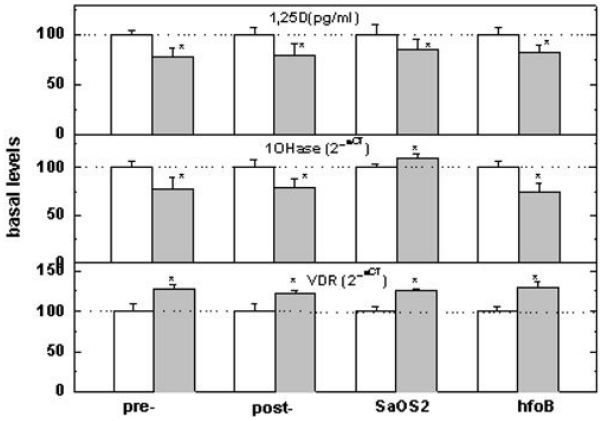

Figure 5b: Modulation by HG (44nM compared to 22nM)) of VDR and 1OHase mRNAs and 1,25D formation in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for analysis as described in Materials and Methods. Results are means ±SEM for triplicate cultures from 5 donors for each group

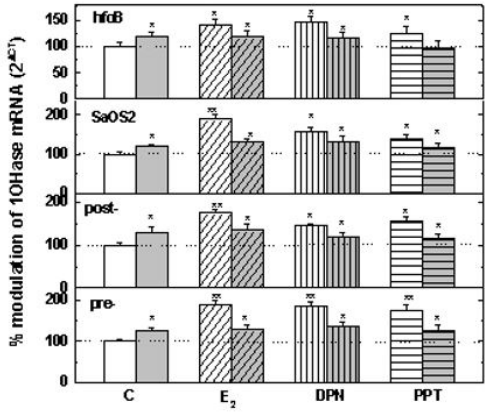

Figure 6a: Modulation by HG (44nM compared to 22nM)) of the modulation by E_2, DPN and PPT of the expression of mRNA for VDR in primary human female derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for real time PCR analysis as described in Materials and Methods. Results are means ±SEM for triplicate cultures from 5 donors for each group. Means of cells grown in high glucose (grey bars) were compared to cells grown in normal glucose (openbars): *, P <0.05; **, P <0.01

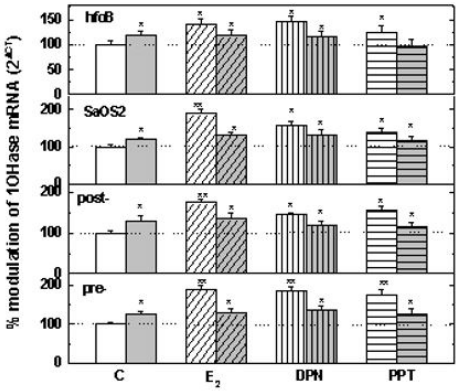

Figure 6b: Modulation by HG (44nM compared to 22nM)) of the modulation by E_2, DPN and PPT of the expression of mRNA for 1OHase in primary human female- derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for real time PCR analysis as described in Materials and Methods. Results are means ±SEM for triplicate cultures from 5 donors for each group. Means of cells grown in high glucose (grey bars) were compared to cells grown in normal glucose (open bars): *, P <0.05; **, P <0.01.

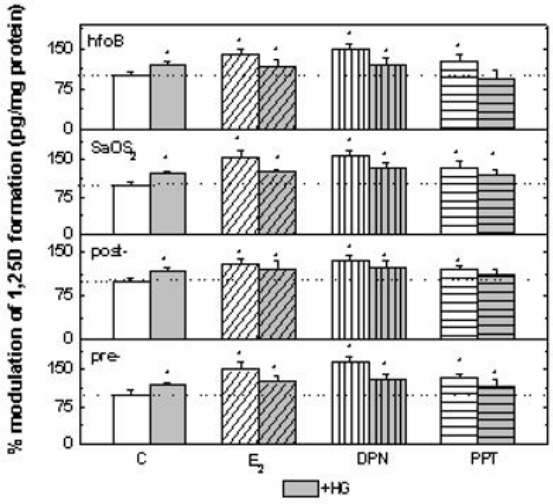

Figure 6c: Modulation by HG (44nM compared to 22nM)) of the stimulation by E_2, DPN and PPT of the Formation of 1,25D in primary human female- derived osteoblasts and SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for analysis as described in Materials and Methods. Results are means ±SEM for triplicate cultures from 5 donors for each group. Means of cells grown in high glucose (grey bars) were compared to cells grown in normal glucose (open bars): *, P <0.05; **, P <0.01.

Expression and Modulation of 12LO and 15LO in Human Female-Derived Osteoblasts by HG

Female-derived bone cells from both age groups or cell lines expressed mRNA for 12LO and 15LO as measured by real time PCR, corrected for RNAse P mRNA. Growing the cells in HG increased the expression of 12LO and 15LO in all cells to different extent (Figure 7b). Cells treated with E_2, DPN or PPT, increased 12LO mRNA in all cells (Figure 7b). HG lowered 15LO mRNA (Figure 8a). In HG the increase of 15LO mRNA expression by the different estrogens was slightly reduced by HG compared to NG (Figure 8b).

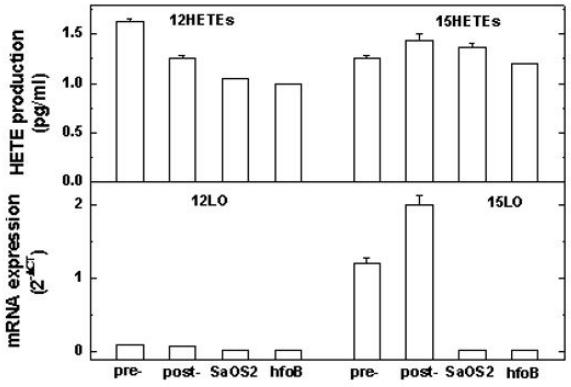

Figure 7a: Basal expression of 12 and 15LO mRNAs and 12 and 15HETE formation in primary human female- derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained, cultured and extracts prepared for analysis as described in Materials and Methods. Results are means ±SEM for triplicate cultures from 5 donors for each group

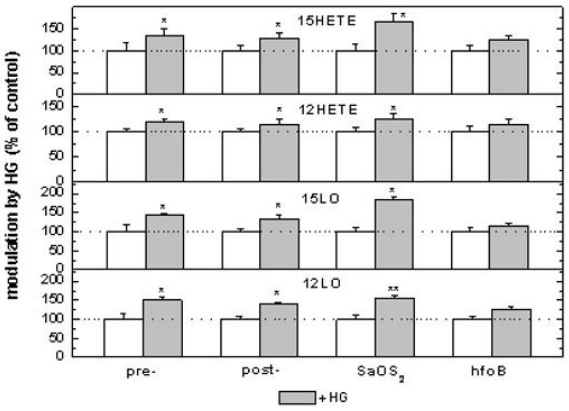

Figure 7b: Modulation by HG (44nM compared to 22nM)) of 12 and 15LO mRNAs and 12 and 15HETE formation in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for analysis as described in Materials and Methods. Results are means ±SEM for triplicate cultures from 5 donors for each group

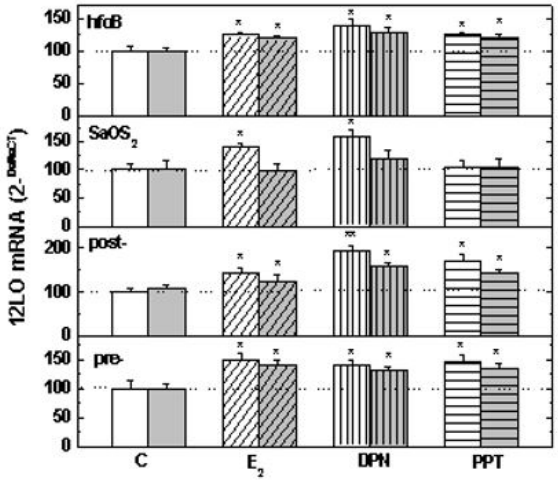

Figure 8a: Modulation by HG (44nM compared to 22nM)) of the stimulation by E_2, DPN and PPT of the expression of mRNA for 12LO in primary human female423 derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for real time PCR analysis as described in Materials and Methods. Results are means ±SEM for triplicate cultures from 5 donors for each group. Means of cells grown in high glucose (grey bars) 427 were compared to cells grown in normal glucose (openbars): *, $P < 0.05$; **, $P < 0.01$

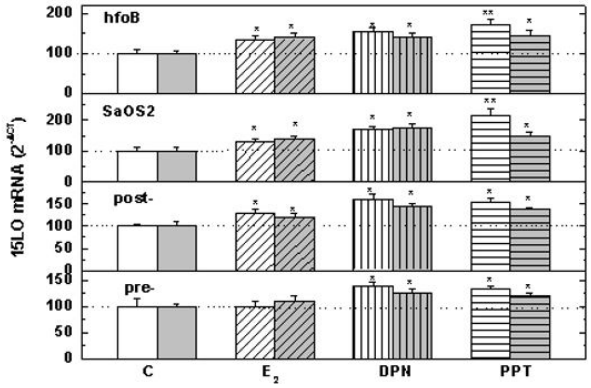

Figure 8b: Modulation by HG (44nM compared to 22nM) of the stimulation by E_2, DPN and PPT of the expression of mRNA for 15LO in primary human female- derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for real time PCR analysis as described in Materials and Methods. Results are means ±SEM for triplicate cultures from 5 donors for each group. Means of cells grown in high glucose (grey bars) were compared to cells grown in normal glucose (open bars): *, P <0.05; **, P <0.01.

Production and Modulation of 12HETE and 15HETE in Human Female-Derived Osteoblasts by HG

Female-derived bone cells from both age groups and cell lines produce 12 and 15HETE (Figure 7a). Growing the cells in HG increased the production of 12 and 15HETE in all cells to different extent (Figure 7b). Cells treated with E_2, DPN or PPT, increased 12 and 15HETEproduction in all cells tested (Figure 8c and d). In HG the increase in 12HETE production by E_2 or DPN in all cells was reduced, whereas the increase by PPT was increased in all cells tested (Figure 8c). In HG the increase in 15HETE production by DPN was reduced, whereas the increase by PPT was increased, but the increase by E_2 was not affected by HG (Figure 8d). This is true to all osteoblasts cells tested.

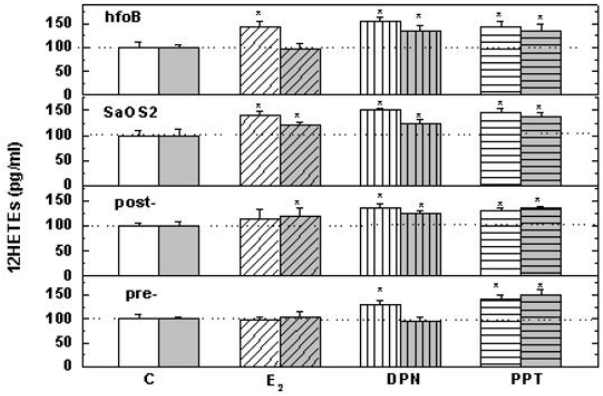

Figure 8c: Modulation by HG (44nM compared to 22nM)) of the stimulation by E_2, DPN and PPT of the formation of 12HETEs in primary human female- derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for analysis as described in Materials and Methods. Results are means ±SEM for triplicate cultures from 5 donors for each group. Means of cells grown in high glucose (grey bars) were compared to cells grown in normal glucose (open bars): *, P <0.05; **, P <0.01.

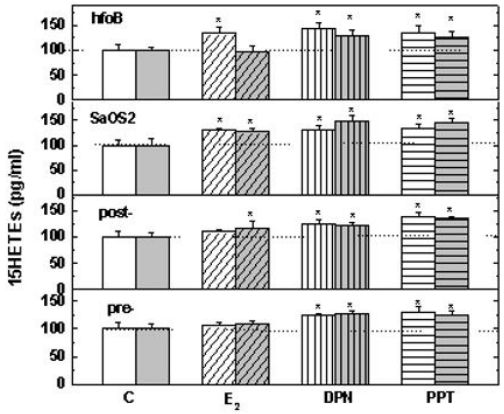

Figure 8d: Modulation by HG (44nM compared to 22nM) of the stimulation by E_2, DPN and PPT of the formation of 15HETEs in primary human female- derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for analysis as described in Materials and Methods. Results are means ±SEM for triplicate cultures from 5 donors for each group. Means of cells grown in high glucose (grey bars) were compared to cells grown in normal glucose (open bars): *, P <0.05; **, P <0.01.

Discussion

The estrogenic compounds tested in our studies can be divided roughly into two classes on the basis of their ER specificity, in human female bone cells. Similarly to E_2, DPN and PPT showed higher stimulation in pre-menopausal than in post-menopausal cells and similar results in SaOS2 and hfoB human female osteoblastic cell lines.

Growing the cells in high glucose concentration (HG; 44mM instead of NG; 22mM) sharpens the ability to distinguish between the groups. First of all, the hyperglycemia increased the constitutive levels of DNA and in CK in all cells except hfoB cell line (Figure 1b). Moreover, the stimulation of DNA and CK by E_2 was abolished by hyperglycemia in both age groups but not in the cell lines, the stimulation of DNA and CK by DPN and PPT was slightly decreased by hyperglycemia in age groups, (figure 2a and b). It is important to note that the constitutive levels of DNA synthesis and CK specific activity were increased by HG in age group bone cells and SaSO2 and hfoB cell lines (figure 4a and b). In order to understand the mechanism of the changes induced by hyperglycemia, we show that the abolition of estrogenic stimulation by hyperglycemia was accompanied in contrast, by increases in mRNA levels of ERα and to less extent in ERβ in female cells tested (Figure 4a and b). This parallels our previous findings (Somjen *et al* 2004a, 2004b), using human vascular smooth muscle cells [17,18]. Attempt to correlate estrogen receptors mRNAs with the changes in nuclear and/or membrane binding failed also in these vascular cells (Somjen *et al* 2004a, 2004b, 2011a) [16-18].

The modulations of ERs by hyperglycemia is a recent addition to the spectrum of changes induced by hyperglycemia, which stimulates the differentiation of osteoblasts and osteoclastsbto produce osteocalcin and alkaline phosphatase.

Bone growth in diabetes which is disturbed in diabetes (He *et al* 200 and Somjen *et al* 2005) is also not enhanced to the same extent by hormone replacement therapy (Consensus opinion of The North American Menopause Society, 2000), and might be the result of lower hip BMD in young women due to their type 1 diabetes (Somjen *et al* 2005); therefore the use of the specific phytoestrogens and their synthetic derivatives, might provide an alternative solution. Bone cells also express VDR and 1OHase which is the enzyme synthesizing the active form of vitamin D metabolite 1,25D (Figure 5a) [9,10,13]. Hypergycemia increased only slightly the VDR expression without affecting 1OHase expression and activity as measured by 1,25D FORMATION (Figure 5b). Whether these changes are leading to bone physiology changes to possible changes in 1,25D is not yet clear. The modulation of VDR and 1OHase as well as 1,25D is not yet clear. The modulation of VDR and 1OHase as 1,25D is an addition to the spectrum of changes induced by hyperglycemia. Recent publications linked 12 and 15LO with bone density.

In the present study we show that the expression of Los in these cells is modulated by (Figure 7a and b). Growing the cells by HG modulated the induction of 12LO mRNA by E2 and DPN, and slightly affected the stimulation of 15LO mRNA by E2, Dpn, and PPT (Figure 8a and b). The expression of these enzymes results in production of 12 and 15HETE. Their production and induction by different estrogens are modulated by HG [19,20].

The exact mechanism of the effect of growing the cells in HG on the responses to estrogens is not yet studied and clear. We believe that exploring agents that are more effective in HG alone and/or in combination with different drugs is required. If these experiments show promising results, we should use different animal models which might lead to human studies.

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