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

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ABSTRACT

Human female-derived cultured osteoblasts (hObs) and human female osteoblastic cell lines (SaOS2 and hfoB) express mRNAs involved in bone biology and physiology, such as estrogen receptors α (ERα) and β (ERβ), 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; 9.0 g/L; 44 mM) compared to normal glucose (NG; 4.5 g/L; 22 mM). HG significantly increased DNA synthesis (DNA) and creatine kinase specific activity (CK). Stimulations of DNA but not of CK by E_2, by 4, 4', 4''-[4-propyl-(1H)-pyrazol-1, 3, 5-triyl] tris-phenol (PPT; ERα specific agonist) or by 2, 3-bis (4-hydroxyphenyl)-propio-nitrile (DPN; ERβ 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 no effect on ERα and 1OHase mRNA expression. The different hormonal treatments modulated the expression 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, VDR, 1OHase, LOs, Estrogens

INTRODUCTION

We have previously studied the effects of estrogens on rat bone physiology 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* [3,4] which contain detectable concentrations of E_2 receptors [5,6]. Estrogens are known for their beneficial effect in osteoporosis [5], characterized by reduction in bone mineral density, leading to fractures after minimal trauma. The biological effects of estrogens in cells and tissues, is initiated by binding to estrogen receptors (ERs). Two ERs have been identified, ERα and ERβ, which differ in their structure and tissue distribution [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 desirable [7].

In human-derived cultured osteoblasts (hObs), we found that E_2 increased cell proliferation and CK specific activity in a gender specific manner [8].

Diabetes is associated with a net loss of bone [9,10], with reduction of new bone formation and decreased bone

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mineral density. In diabetic mice the up-regulation of specific transcription factors is attenuated, resulting in deficiency in conversion of mesenchymal cells to osteoblasts [9,10].

We have found previously that E_2 and other estrogenic compounds stimulate also vitamin D (VDR) and 1OHase mRNA expression and activity, measured by 1α, 25 hydroxy vitamin D_3 (1,25D) formation [11-13], as well as lipoxygenase mRNA (12 and 15LO) expression and activity, measured by HETE formation (12 and 15HETE) [13,14].

We now analyzed the effects of high glucose (HG) 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 SaSO2 and hfoB, which is relevant to the important factors existing in diabetes. The compounds analyzed were E2 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 E2, DPN and PPT.
2. Modulation by NG and HG of VDR and 1OHase basal expression as well as 1,25D formation and their responses to treatment with E2, DPN and PPT.
3. Modulation by NG and HG of 12 and 15 LO basal expression as well as 12 and 15HETE formation and their responses to treatment with E2, DPN and PPT.

MATERIALS AND METHODS

Reagents

All reagents used were of analytical grade. E_2, DPN (ERβ specific agonist, 4, 4', 4''-[4-propyl-(1H)-pyrazol-1, 3, 5-triyl] tris-phenol), PPT (ERα specific agonist; 2, 3-bis 4-hydroxyphenyl)-propionitrile) 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) were healthy, non-osteoporotic and not receiving hormonal replacement treatment. Two groups were defined: pre-menopausal women, ranging between 37-55 years old (n=5) and 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 [8]. Cell outgrowth from bone explants was apparent after 6-10 days. 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 nM (9.0 g/L), whereas glucose concentration in the regular medium (NG) was 22 nM (4.5 g/L).
2. SaSO2 and hfoB human female derived bone cell lines were obtained from ATCC and were grown as instructed.

Hormonal treatment

At sub-confluence cells were treated with 30 nM E_2, 300 nM DPN or 3 μM PPT for 24 h, 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 14000x g for 5 min at 4°C in an Eppendorf micro-centrifuge. CK specific activity 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 [8].

Assessment of DNA synthesis

Cells were grown until sub-confluence and then treated with various hormones as indicated. 22 h later, ^{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 then 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 assayed [1].

Determination of mRNA for ERα, ERβ, VDR, 25 hydroxy vitamin D_3 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 described for the different parameters [12,14-16].

Assessment of 1OHase activity

1OHase activity was assessed by measuring 1,25 $(OH)_2D_3$ (1,25D) generated in hObs within 60 min after the addition of 25(OH)D_3 (200 ng/ml) to cultures, using 1,25D ^{125}I RIA kit from Dia Sorin, Mn, USA. Protein was assayed by Coomassie brilliant blue dye binding, using BSA as standard.

Assessment of 12 and 15LO activity

12 lipoxygenase (12LO) and 15 lipoxygenase (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 [13].

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)**. Growing cells in HG increased basal level of DNA synthesis (DNA) in pre-menopausal osteoblasts by 153 ± 20% and in post-menopausal osteoblasts by 165 ± 13% **(Figure 1b)**, in SaOS2 by 200 ± 12% but not in hfOB by 108 ± 8% **(Figure 1b)**. Female derived hObs treated with E_2, DPN or PPT for 24 h, showed a significant increase in CK **(Figure 2b)**, in both age groups **(Figure 2b)** and both cell lines. The response of pre-menopausal cells was higher than post- and more than in SaOS2 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 PPT in cells from both age groups and in both cell lines **(Figure 2a)**.

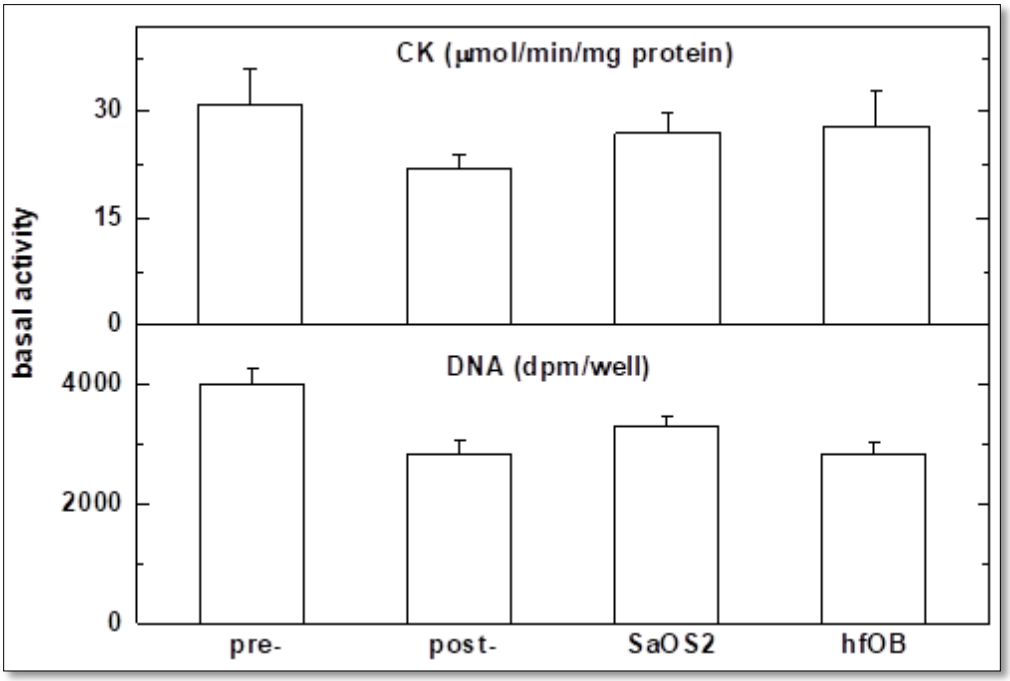

Figure 1a. Basal levels of DNA synthesis and CK specific activities in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described in Materials and Methods. Results are means ± SEM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

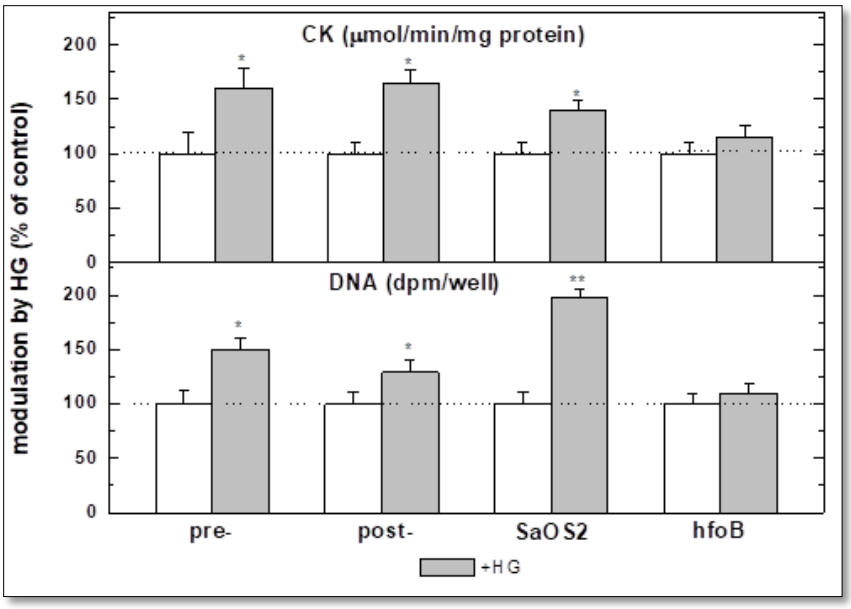

Figure 1b. Modulation by HG (44 nM compared to 22 nM)) of basal levels of DNA synthesis and CK specific activities in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described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

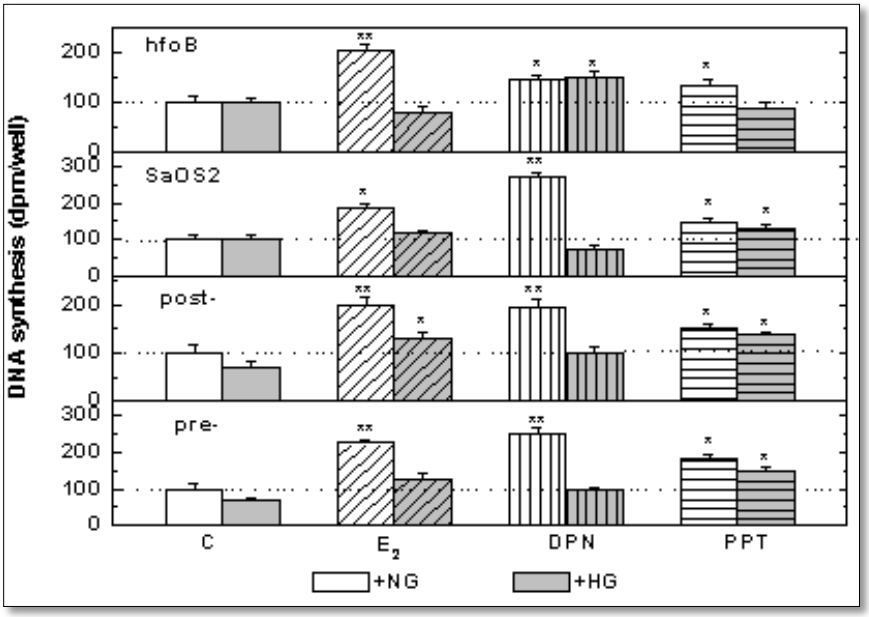

Figure 2a. Modulation by HG (44 nM compared to 22 nM) of the stimulation by E_2, DPN and PPT of DNA synthesis in primary human female-derived osteoblasts as well as SaOS2 as well as hfoB human female bone cell lines. Bone cells were obtained, cultured, treated and extracts prepared for analysis as described 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): *, $P<0.05$; **, $P<0.01$.

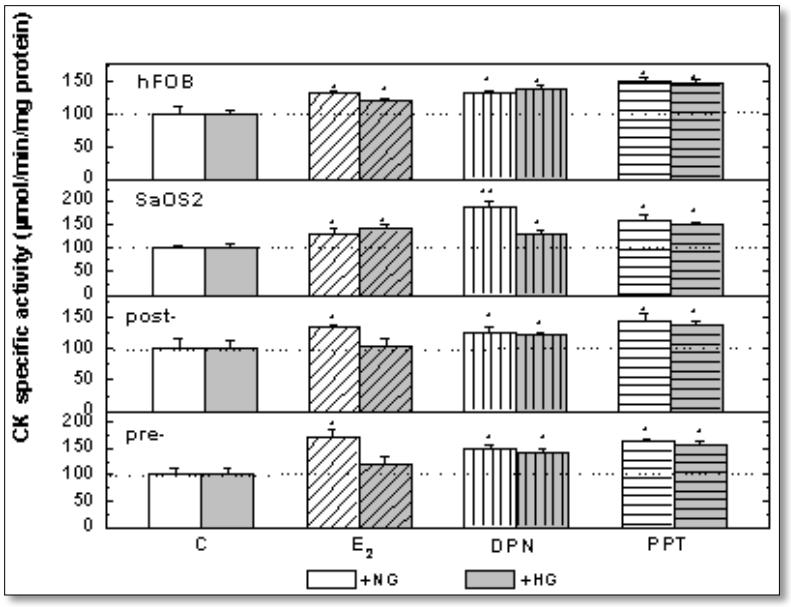

Figure 2b. Modulation by HG (44 nM compared to 22 nM) of the stimulation by E_2, DPN and PPT of CK specific activity in primary human female-derived osteoblasts and SaOS2 as well as hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described 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 female 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 **(Figure 4a)**, in all cells with different patterns of modulations of these effect by HG **(Figure 4a)**. Similar results were obtained also when ERβ was assayed **(Figure 4b)**.

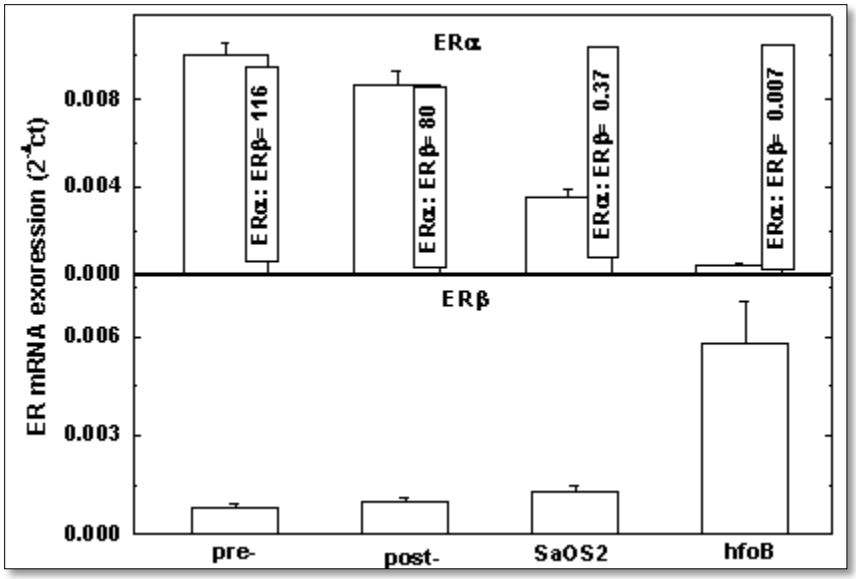

Figure 3a. Basal expression of ERα and ERβ mRNAs in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described in Materials and Methods. Results are means ± SEM for triplicate cultures from 5 donors for each group.

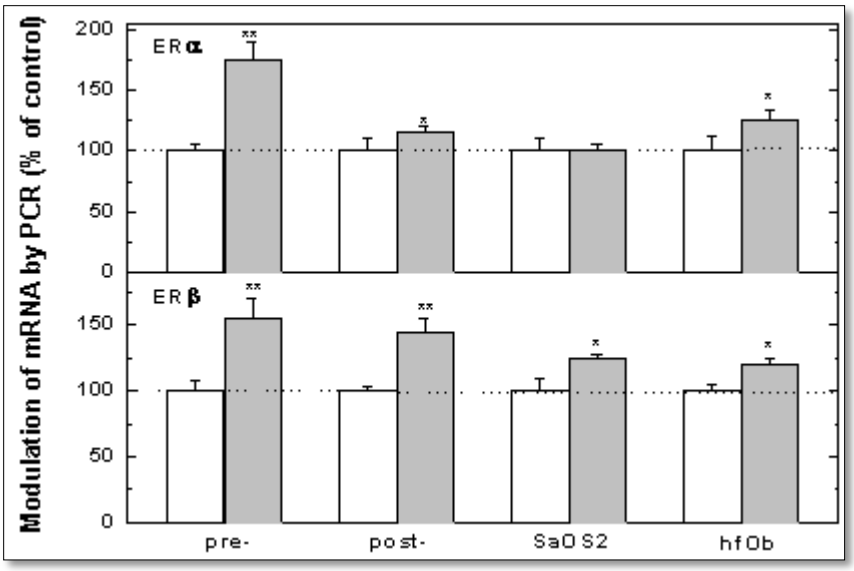

Figure 3b. Modulation by HG (44 nM compared to 22 nM)) on basal expression of ERα and ERβ mRNAs in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described in Materials and Methods. Results are means ± SEM for triplicate cultures from 5 donors for each group.

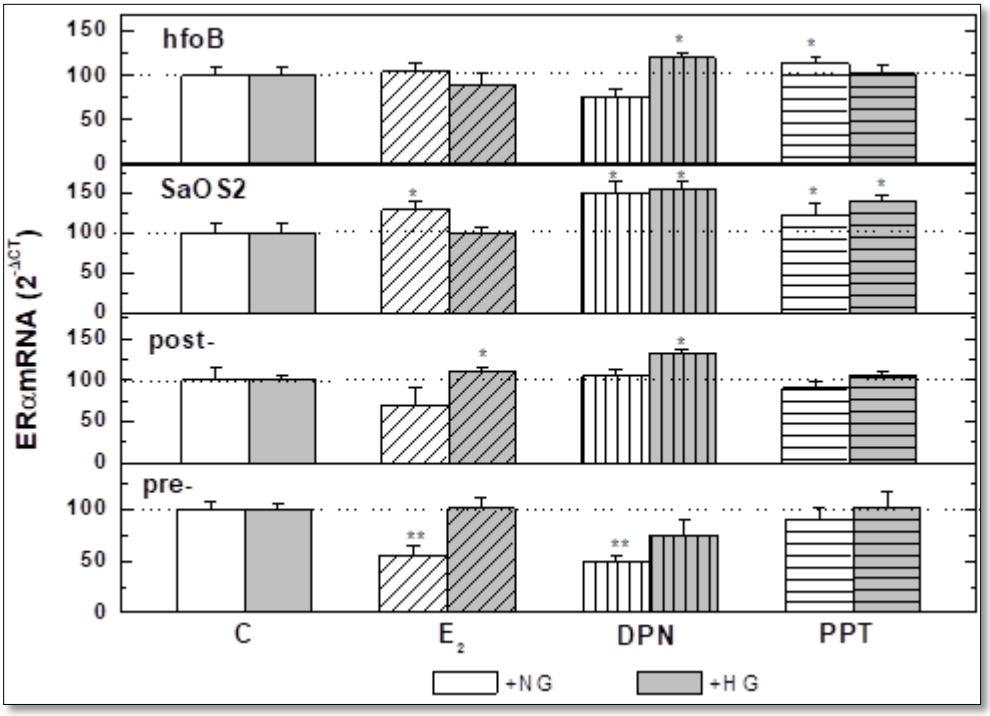

Figure 4a. Modulation by HG (44 nM compared to 22 nM) of the modulation by E_2, DPN and PPT of the expression of mRNA for ERα in primary human female-derived osteoblasts and SaOS2 as well as hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for real time PCR 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.

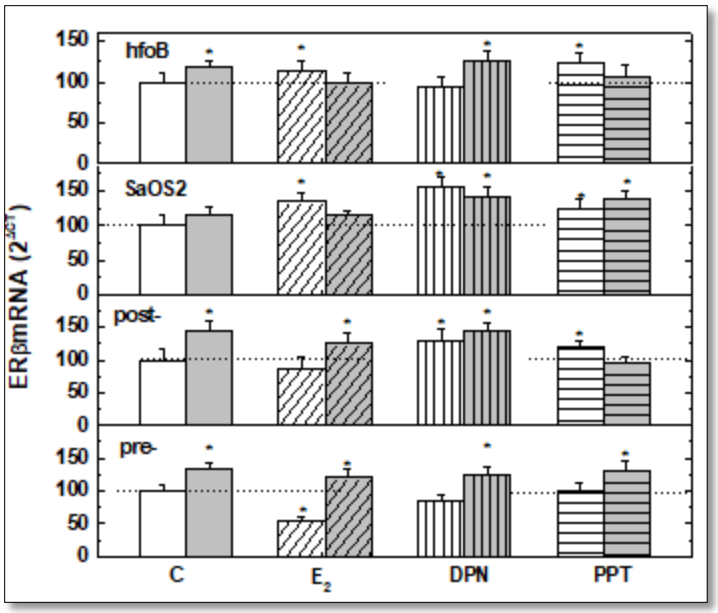

Figure 4b. Modulation by HG (44nM compared to 22nM)) of the modulation by E_2, DPN and PPT of the expression of mRNA for ERβ in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for real time PCR 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 female age groups and cell lines expressed mRNA for VDR and 25 hydroxy vitamin D_3 1-α hydroxylase (1OHase) as measured by real time PCR, corrected for RNAse P mRNA and also produced $1,25(OH)_2D_3$ (1,25D) as measured by radio-immunoassay **(Figure 5a)**. Growing the cells in HG decreased the expression of 1OHase as well as 1,25D production respectively, in both age groups and SaSO2 and hfoB **(Figure 5b)**. While growing the cells in HG increased VDR in all cells tested **(Figure 5b)**.

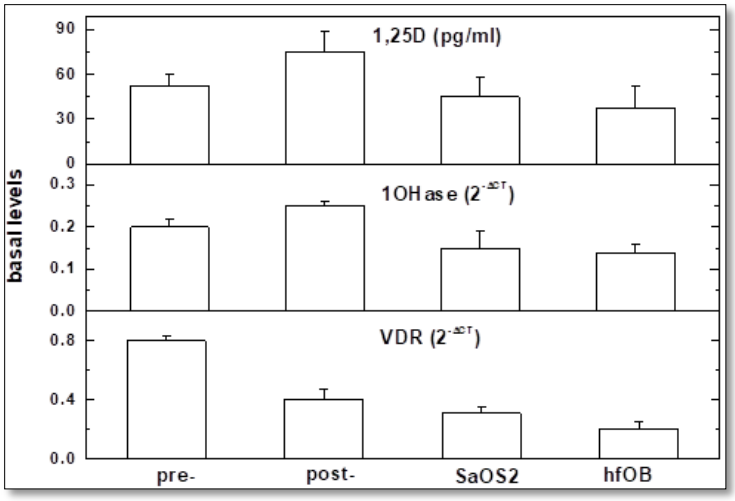

Figure 5a. Basal expression of VDR and 1OHase mRNAs and 1,25D formation in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described in Materials and Methods. Results are means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

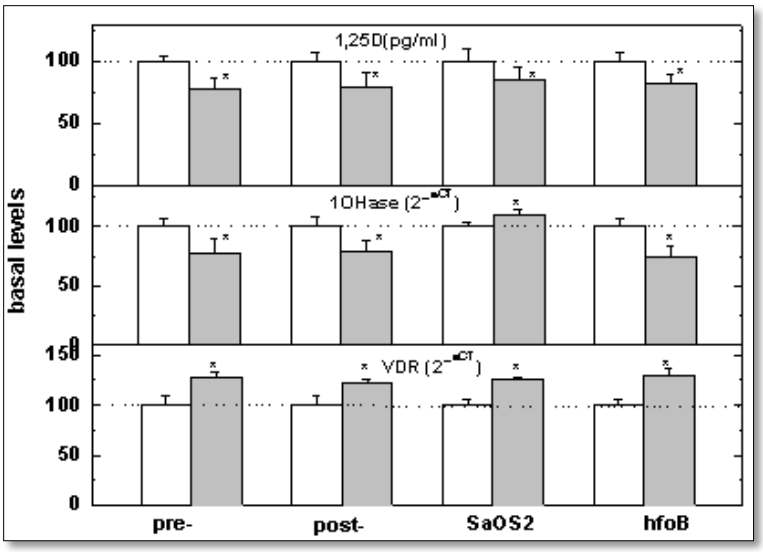

Figure 5b. Modulation by HG (44 nM compared to 22 nM)) of VDR and 1OHase mRNAs and 1,25D formation in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described in Materials and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

Female derived hObs treated with E_2, DPN or PPT, showed a significant modulation in VDR **(Figure 6a)**. In all cells HG decreased its expression **(Figure 6a)**. Similar results were obtained when 1OHase was assayed for both mRNA expression and its activity as measured by 1,25D production **(Figures 6b and 6c)**.

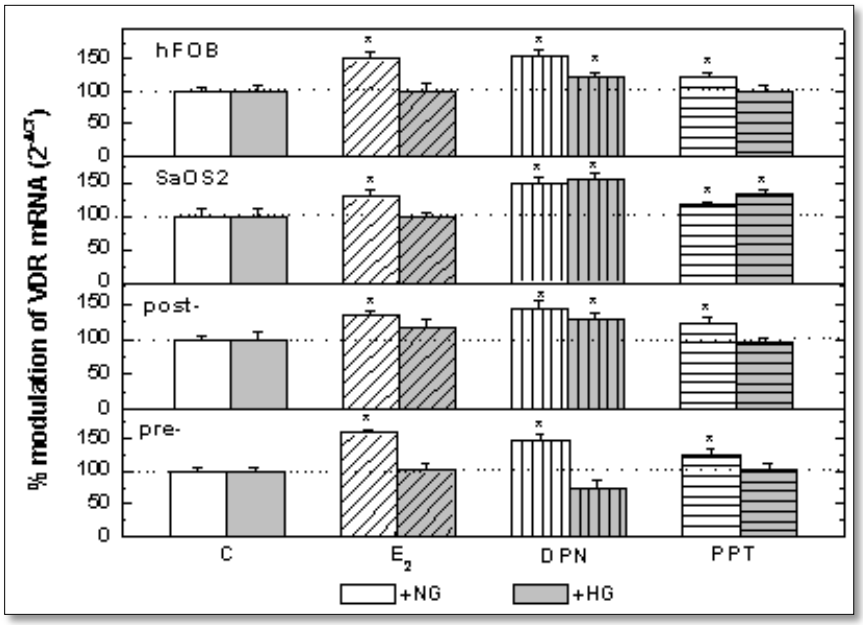

Figure 6a. Modulation by HG (44 nM compared to 22 nM)) of the modulation by E_2, DPN and PPT of the expression of mRNA for VDR in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for real time 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 (open bars): *, $P<0.05$; **, $P<0.01$.

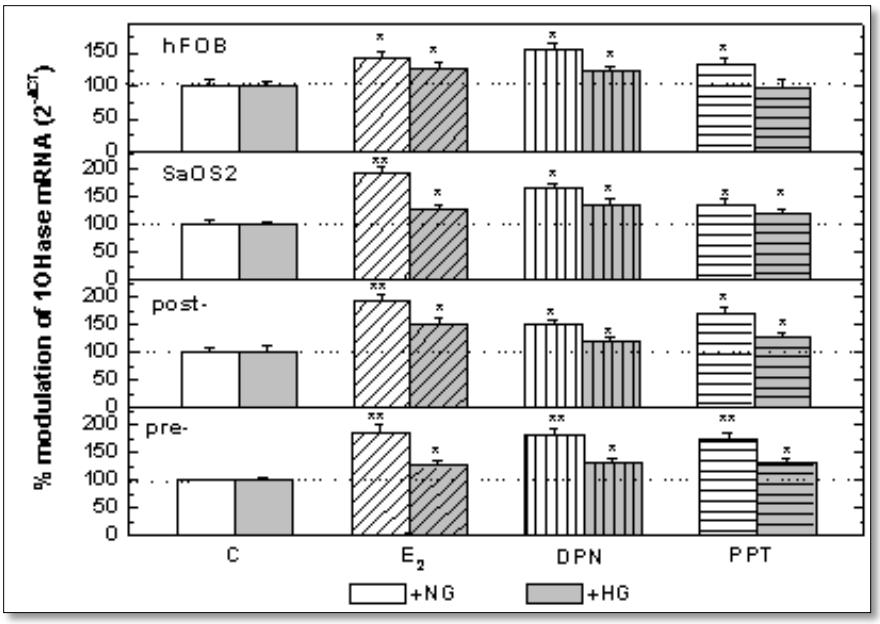

Figure 6b. Modulation by HG (44 nM compared to 22 nM)) of the modulation by E_2, DPN and PPT of the expression of mRNA for 1OHase in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for real time 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): *, $P<0.05$; **, $P<0.01$.

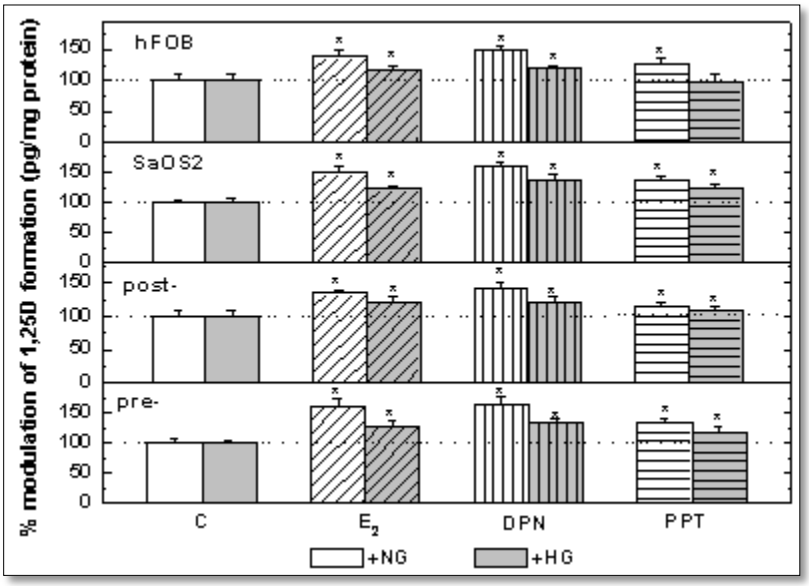

Figure 6c. Modulation by HG (44 nM compared to 22 nM)) of the stimulation by E_2, DPN and PPT of the formation of 1,25D in primary human female-derived osteoblasts and SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described in 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 female age groups or SaOS2 and hfoB cell lines expressed mRNA for 12LO and 15LO as measured by real-time PCR **(Figure 7a)**. Growing the cells in HG increased the expression of 12LO and 15LO in all cells to different extent **(Figure 7b)**. Female derived hObs treated with E_2, DPN or PPT increased 12LO mRNA expression, in all cells **(Figure 7b)**. Growing the cells in HG

lowered 12LO mRNA, increased expression by estrogenic compounds tested **(Figure 8a)**. Treatment with E_2 or DPN as well as PPT in NG increased the 15LO mRNA expression **(Figure 8b)**. In HG, the increase in 15LO mRNA expression by the different estrogens was slightly reduced by HG compared to NG **(Figure 8b)**.

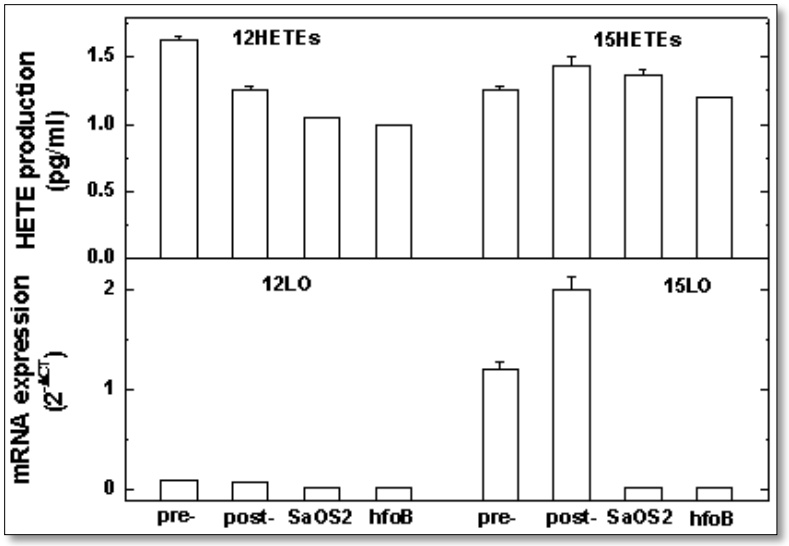

Figure 7a. Basal expression of 12 and 15LO mRNAs and 12 and 15HETE formation in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained, cultured and extracts prepared for analysis as described in Materials and Methods. Results are means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

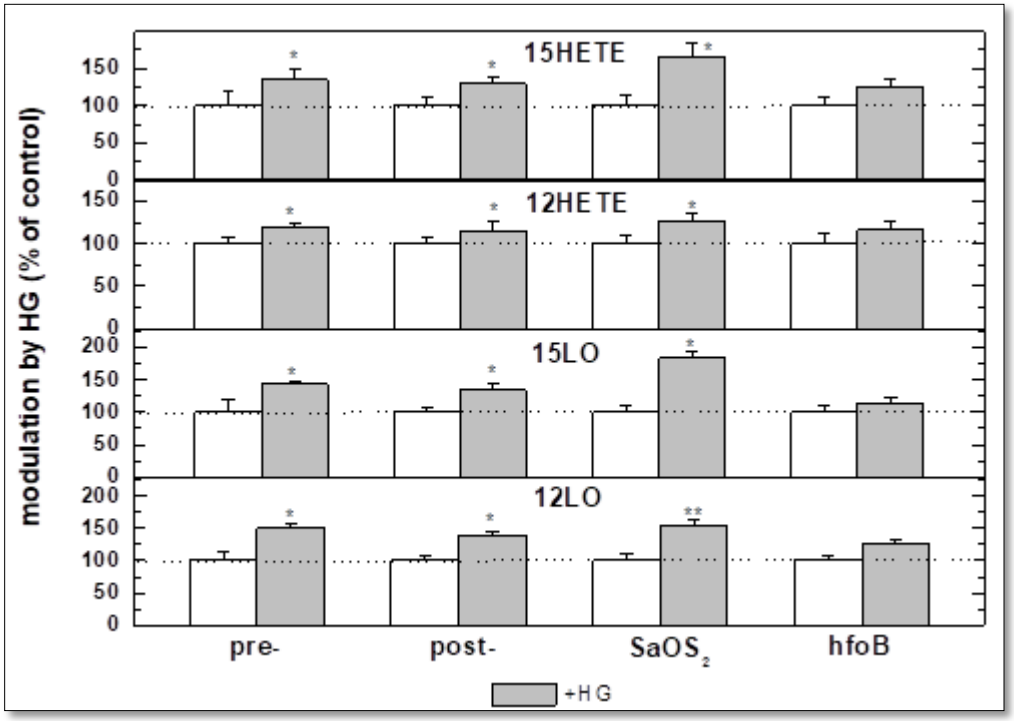

Figure 7b. Modulation by HG (44 nM compared to 22 nM)) of 12 and 15LO mRNAs and 12 and 15HETE formation in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described in Materials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

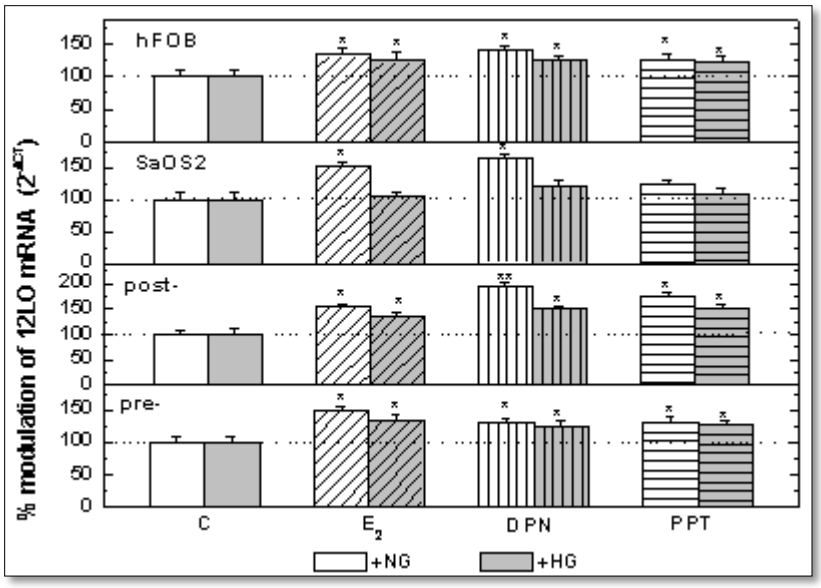

Figure 8a. Modulation by HG (44 nM compared to 22 nM)) of the stimulation by E_2, DPN and PPT of the expression of mRNA for 12LO in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for real time 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): *, $P<0.05$; **, $P<0.01$.

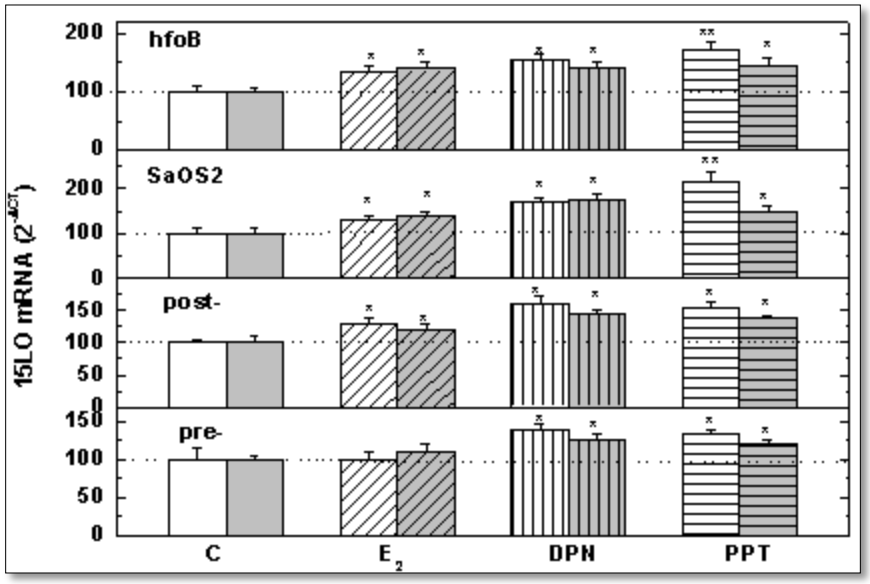

Figure 8b. Modulation by HG (44 nM compared to 22 nM)) of the stimulation by E_2, DPN and PPT of the expression of mRNA for 15LO in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for real time 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): *, $P<0.05$; **, $P<0.01$.

Modulation of the production of 12HETE and 15HETE in human female-derived osteoblasts by HG

Female-derived bone cells from both female age groups and SaOS2 or hfoB cell lines produced 12HETE and 15HETE **(Figure 7a)**. Growing the cells in medium containing HG increased the production of 12HETE and 15HETE in all cells to different extent **(Figure 7b)**. Female derived hObs treated with E_2, DPN or PPT increased 12HETE and 15HETE production, in all cells tested **(Figures 8c and 8d)**. In HG, the increase in 12HETE production by E_2 or DPN was reduced, whereas the increase by PPT was up-regulated

(Figure 8c). In HG, the increase in 15HETE production by DPN was reduced, while the increase by PPT was up-regulated, but the increase by E_2 was not affected by HG **(Figure 8d)**.

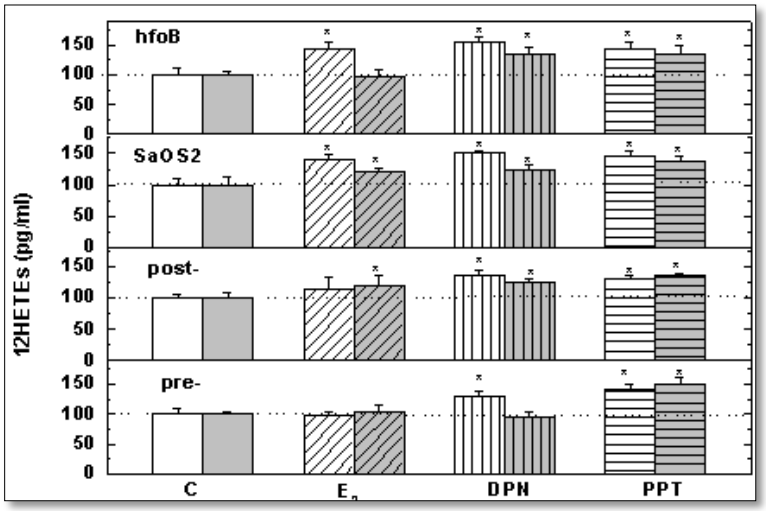

Figure 8c. Modulation by HG (44nM compared to 22nM)) of the stimulation by E_2, DPN and PPT of the formation of 12HETEs in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described 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): *, $P<0.05$; **, $P<0.01$

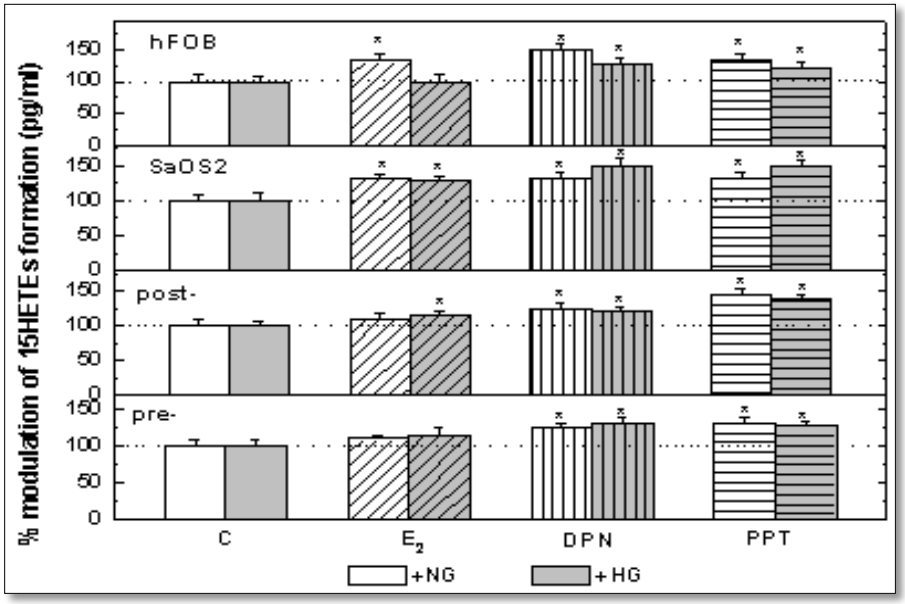

Figure 8d. Modulation by HG (44 nM compared to 22 nM) of the stimulation by E_2, DPN and PPT of the formation of 15HETEs in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described 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 AND CONCLUSION

The estrogenic compounds tested in our studies can be divided into classes based on 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; 44 mM instead of NG; 22 mM) sharpens the ability to distinguish between the groups. First of all, the

hyperglycemia increased the constitutive levels of DNA and of 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 both age groups **(Figures 2a and 2b)**. 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 **(Figures 4a and 4b)**. 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 all female cells tested **(Figures 4a and 4b)**. This parallels our previous findings [17,18], using human vascular smooth muscle cells. Attempt to correlate estrogen receptors mRNAs with the changes in nuclear and/or membrane binding failed also in the human vascular smooth muscle cells [13,17,18].

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

Bone growth which is disturbed in diabetes [9,14] 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 [14]; therefore the use of the specific phytoestrogens and their synthetic derivatives that we are currently using, might provide an alternative solution. Bone cells also express VDR and 1OHase which is the enzyme synthesizing the active of vitamin D metabolite of the 1, 25 $(OH)_2D_3$ (1,25D) **(Figure 5a)**. Hyperglycemia 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 due to possible changes in 1,25D is not yet clear. The modulation of VDR and 1OHase as well as 1,25D, is an addition to the spectrum of changes induced by hyperglycemia.

Recent publications linked 15LO and 12LO (platelet type) to bone density. In the present study we show that the expression of the LOs in these cells **(Figure 7a)** is modulated by growing the cells in HG **(Figure 7b)**. 12LO mRNA is increased by HG by 55% and 15LO mRNA by 84% **(Figure 7b)**. Growing the cells in HG modulated the induction of 12LO mRNA by E_2 and DPN **(Figure 8a)** and slightly affect the stimulation of 15LO mRNA by E_2, DPN and PPT **(Figure 8b)**. The expression of these enzymes results in the ability of bone cells to produce and secrete 12HETE and 15HETE, the products of LOs. 12HETE production is increased by growing the cells in high glucose **(Figure 8c)**. Growing the cells in HG decreased the induction of 15HETE by E_2 and DPN but increased its induction by PPT **(Figure 8d)**. Growing the cells in HG did not affect the stimulation of 15HETE by E_2, but slightly affected the response to DPN and PPT **(Figure 8d)**.

The exact mechanism of the effects of growing the cells in HG in the growth medium on bone cell responses to estrogenic compounds is yet to be investigated and its relationship to human physiology is not yet clear. We believe that we should explore agents that are more effective in HG conditions alone and/or a combination of different drugs which might be less affected by hyperglycemia. If these experiments show promising results, we will analyze animal models that might lead to human studies.

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