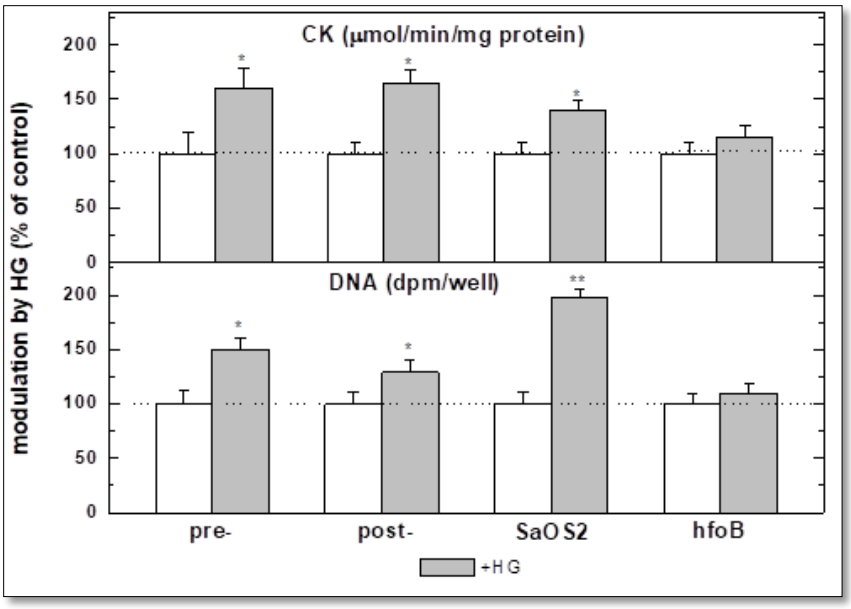


**Figure 1b.** Modulation by HG (44 nM compared to 22 nM)) of basal levels of DNA synthesis and CK specific activities in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described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

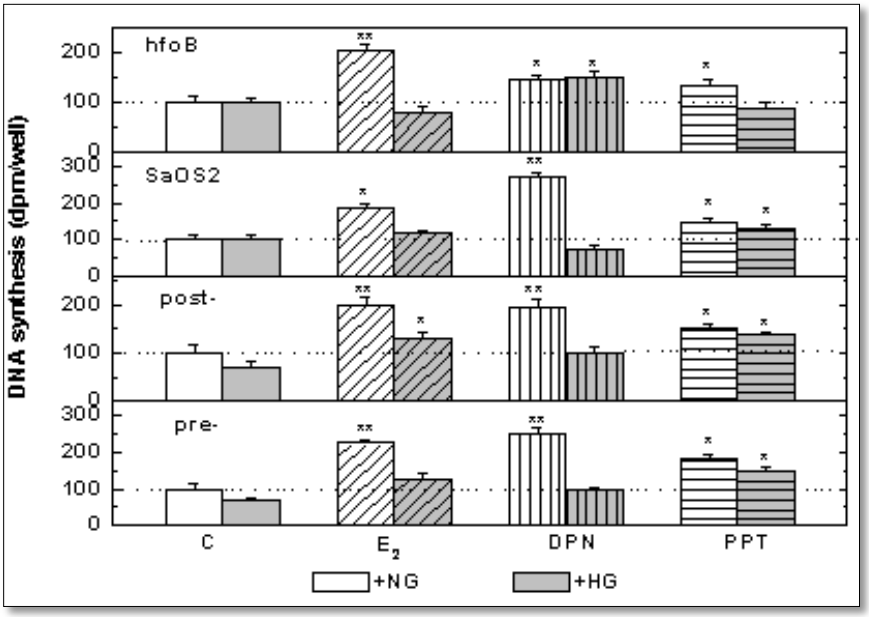


**Figure 2a.** Modulation by HG (44 nM compared to 22 nM) of the stimulation by $E_2$, DPN and PPT of DNA synthesis in primary human female-derived osteoblasts as well as SaOS2 as well as hfoB human female bone cell lines. Bone cells were obtained, cultured, treated and extracts prepared for analysis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

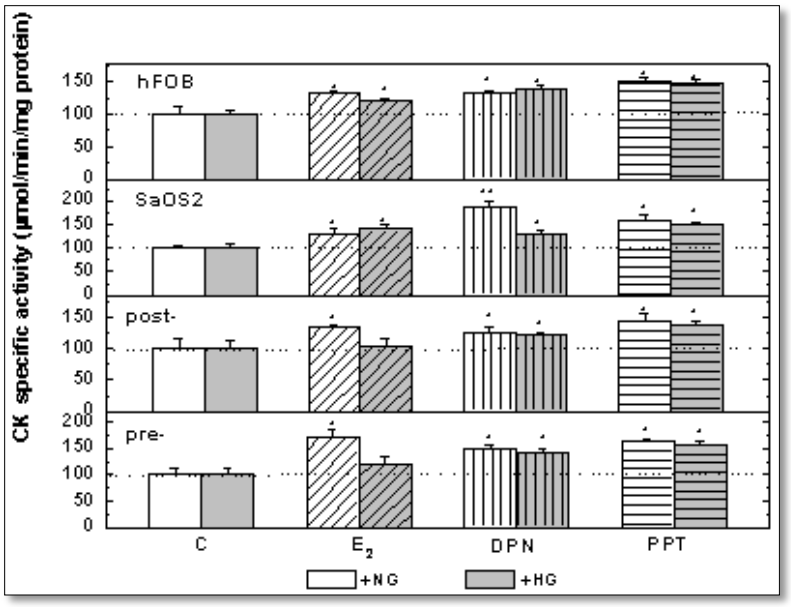


**Figure 2b.** Modulation by HG (44 nM compared to 22 nM) of the stimulation by $E_2$, DPN and PPT of CK specific activity in primary human female-derived osteoblasts and SaOS2 as well as hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described 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)**.

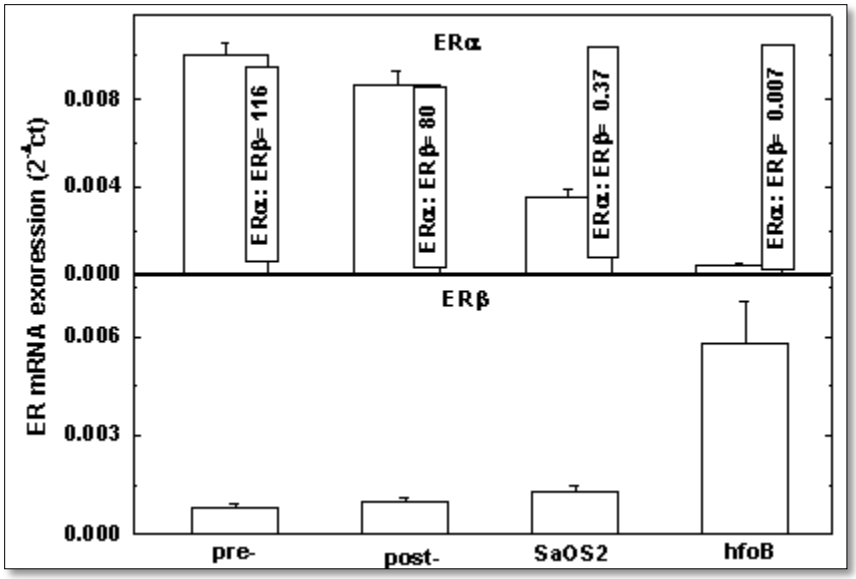


**Figure 3a.** Basal expression of ERα and ERβ mRNAs in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described in Materials and Methods. Results are means ± SEM for triplicate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

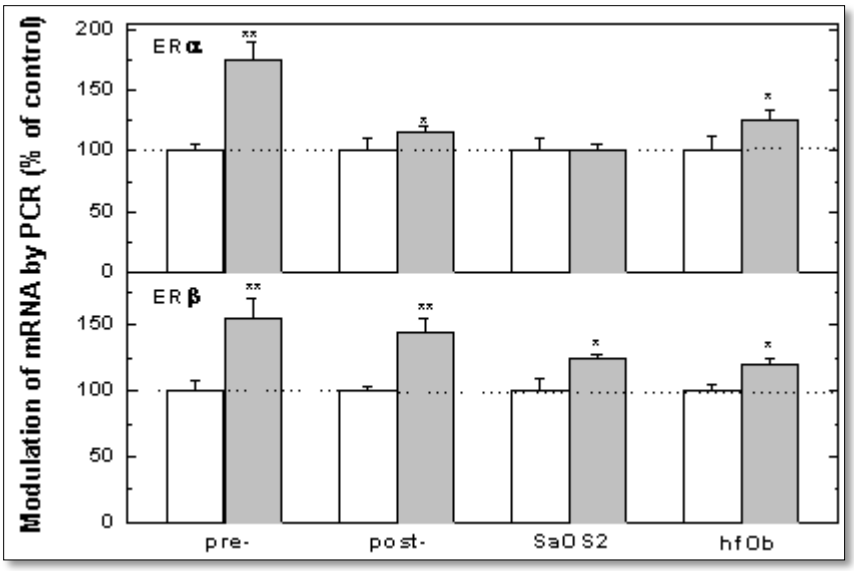


**Figure 3b.** Modulation by HG (44 nM compared to 22 nM)) on basal expression of ERα and ERβ mRNAs in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described in Materials and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

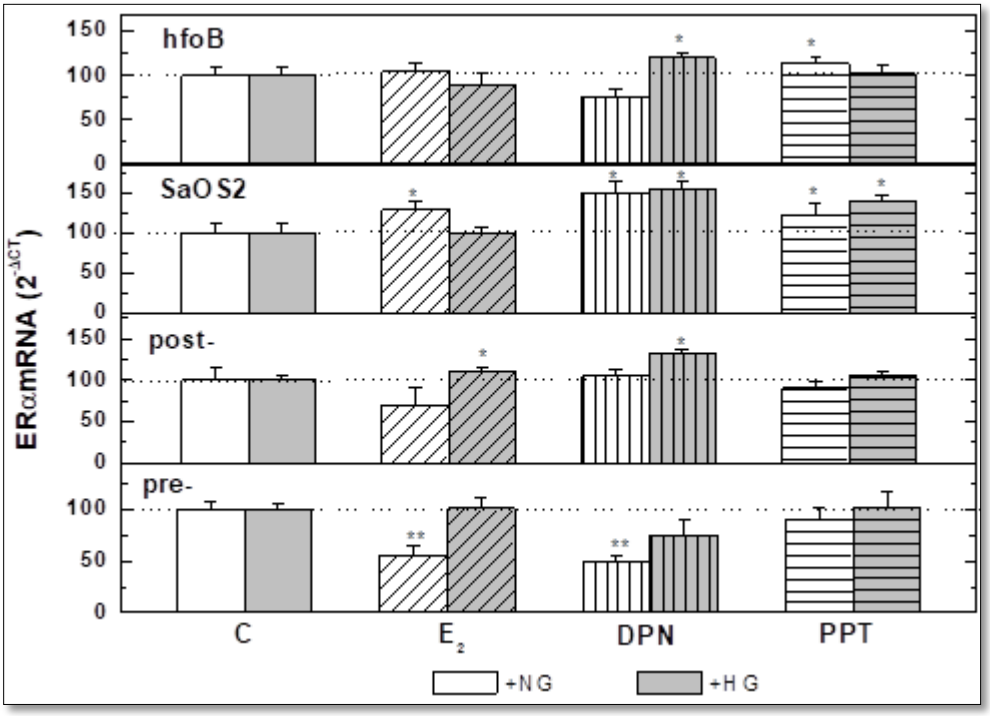


**Figure 4a.** Modulation by HG (44 nM compared to 22 nM) of the modulation by $E_2$, DPN and PPT of the expression of mRNA for ERα in primary human female-derived osteoblasts and SaOS2 as well as hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for real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

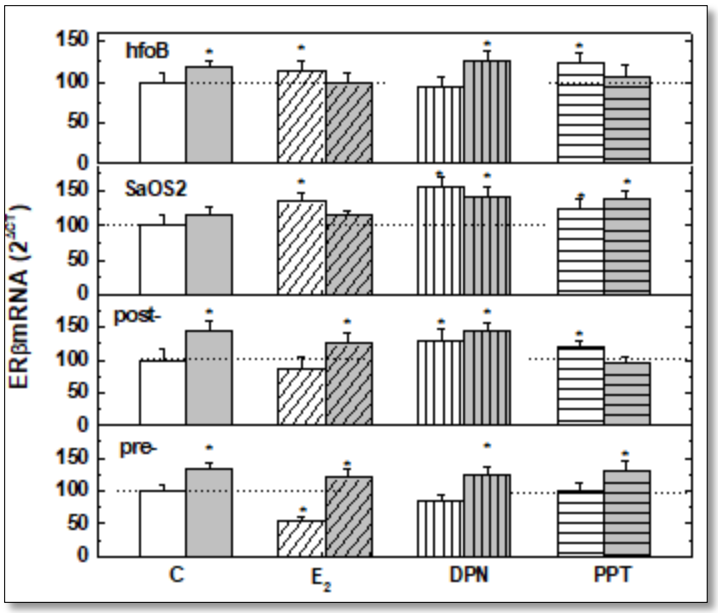


**Figure 4b.** Modulation by HG (44nM compared to 22nM)) of the modulation by $E_2$, DPN and PPT of the expression of mRNA for ERβ in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for real time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

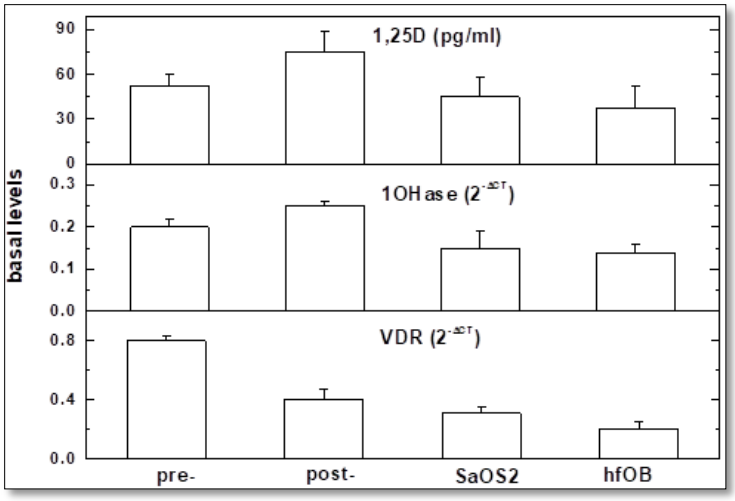


**Figure 5a.** Basal expression of VDR and 1OHase mRNAs and 1,25D formation in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described in Materials and Methods. Results are means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

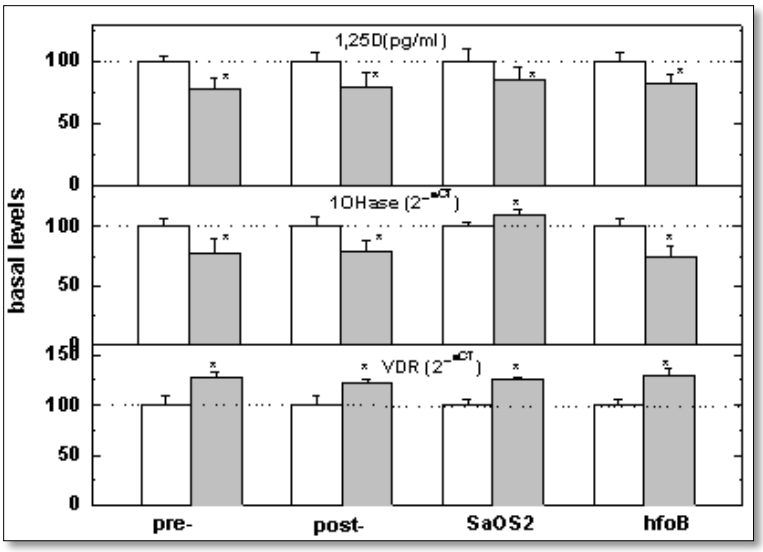


**Figure 5b.** Modulation by HG (44 nM compared to 22 nM)) of VDR and 1OHase mRNAs and 1,25D formation in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described in Materials 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)**.

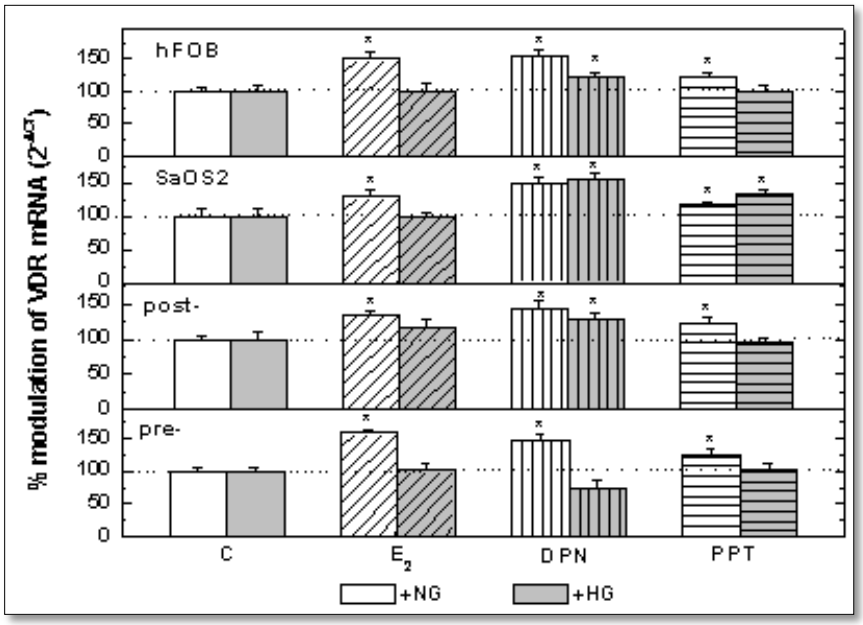


**Figure 6a.** Modulation by HG (44 nM compared to 22 nM)) of the modulation by $E_2$, DPN and PPT of the expression of mRNA for VDR in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for real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

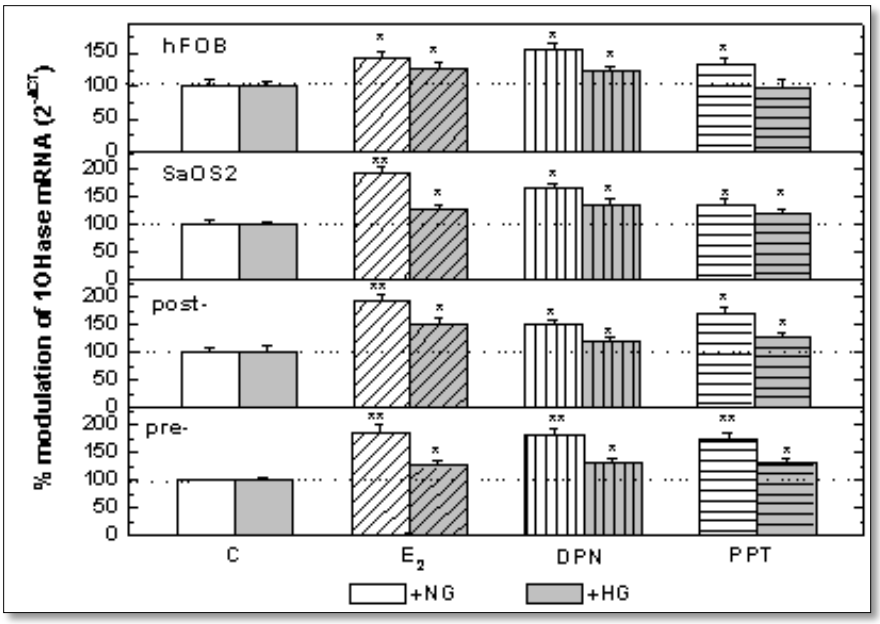


**Figure 6b.** Modulation by HG (44 nM compared to 22 nM)) of the modulation by $E_2$, DPN and PPT of the expression of mRNA for 1OHase in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for real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

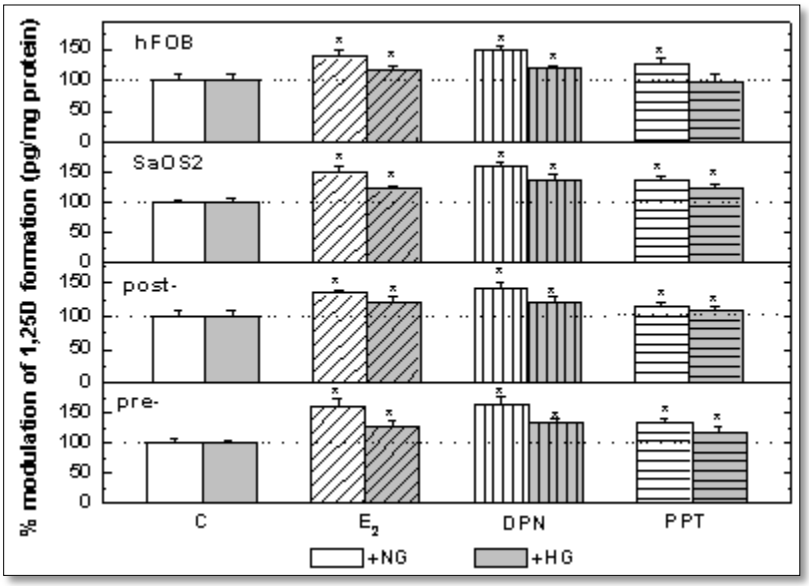


**Figure 6c.** Modulation by HG (44 nM compared to 22 nM)) of the stimulation by $E_2$, DPN and PPT of the formation of 1,25D in primary human female-derived osteoblasts and SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described 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)**.

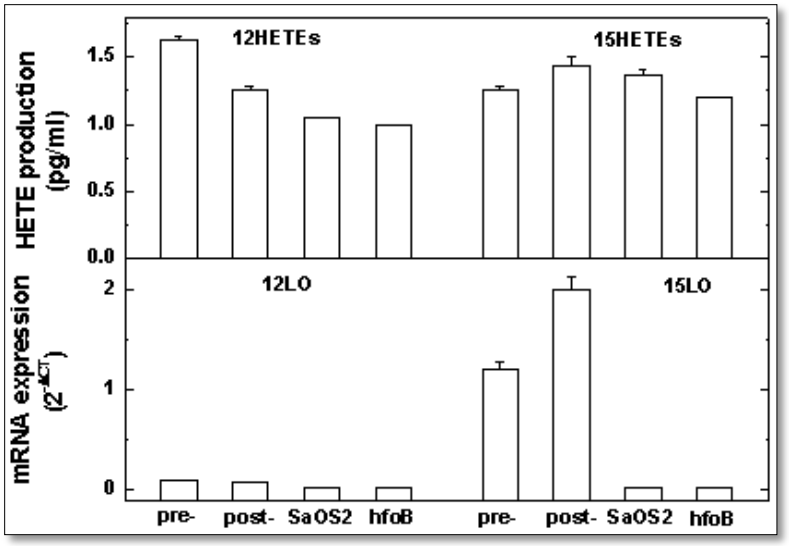


**Figure 7a.** Basal expression of 12 and 15LO mRNAs and 12 and 15HETE formation in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained, cultured and extracts prepared for analysis as described in Materials and Methods. Results are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

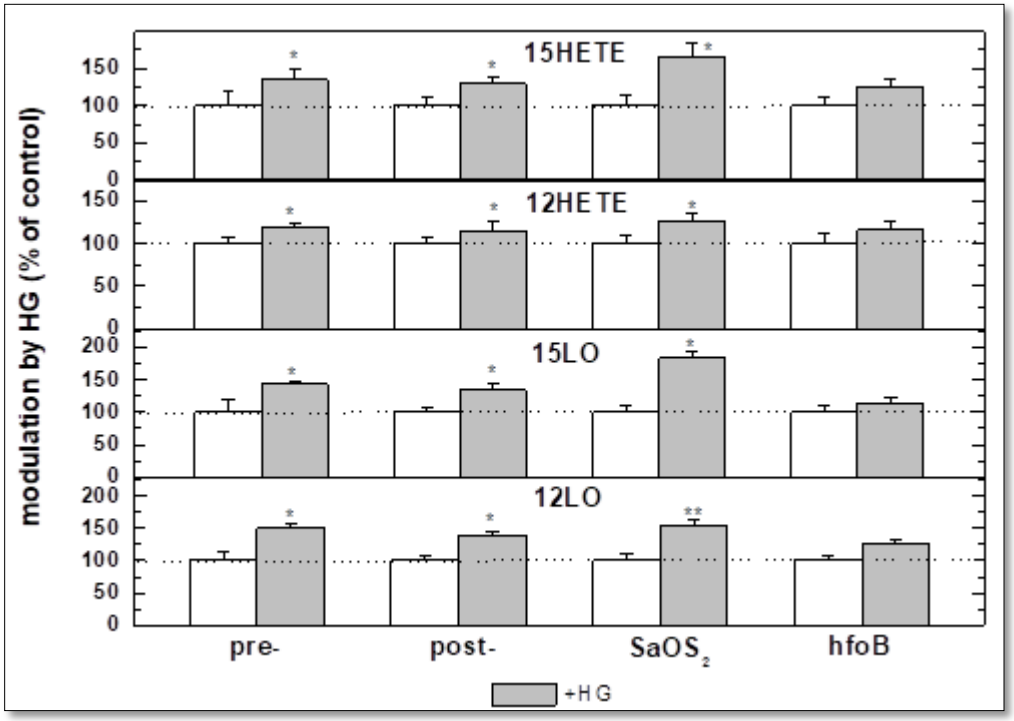


**Figure 7b.** Modulation by HG (44 nM compared to 22 nM)) of 12 and 15LO mRNAs and 12 and 15HETE formation in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described in Materials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

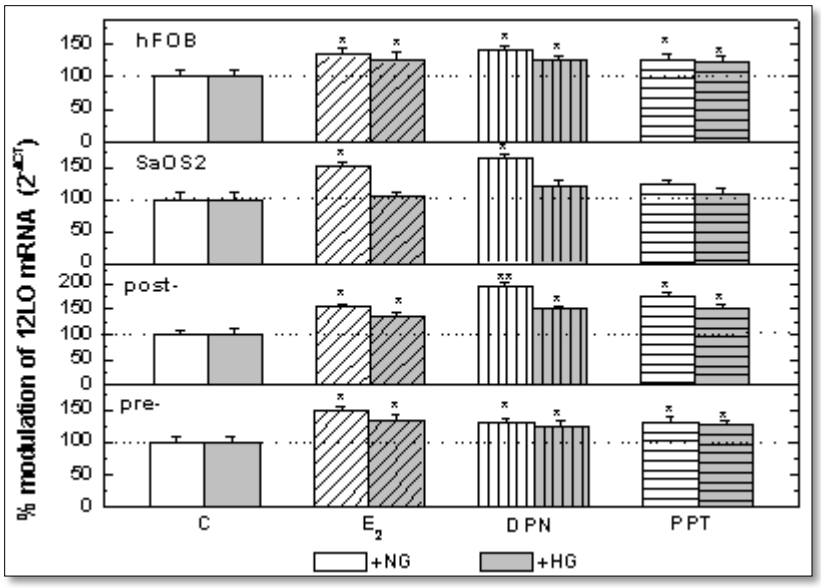


**Figure 8a.** Modulation by HG (44 nM compared to 22 nM)) of the stimulation by $E_2$, DPN and PPT of the expression of mRNA for 12LO in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for real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

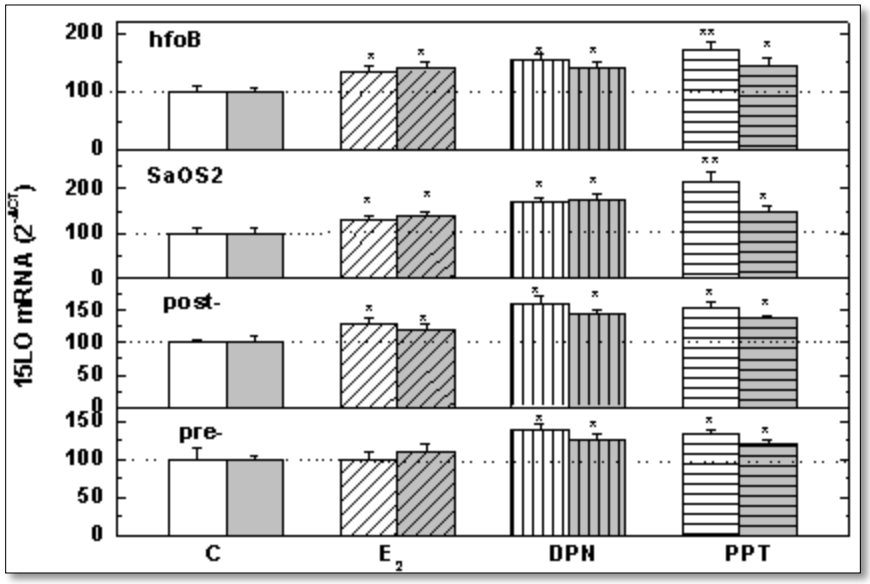


**Figure 8b.** Modulation by HG (44 nM compared to 22 nM)) of the stimulation by $E_2$, DPN and PPT of the expression of mRNA for 15LO in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for real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

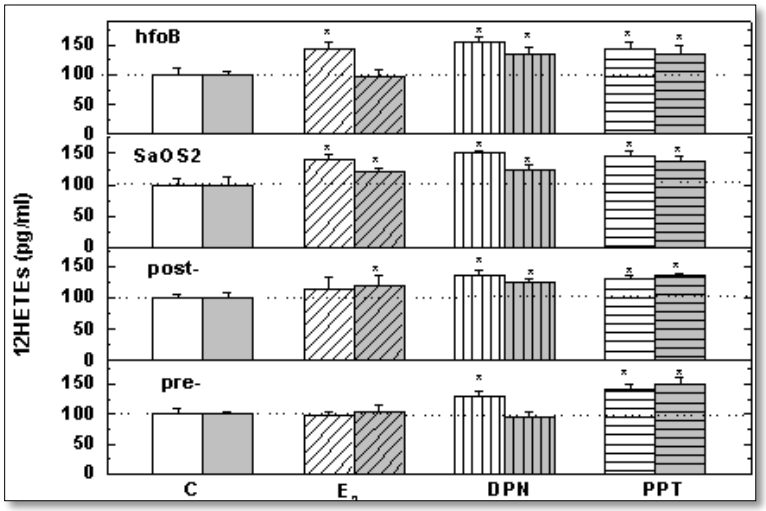


**Figure 8c.** Modulation by HG (44nM compared to 22nM)) of the stimulation by $E_2$, DPN and PPT of the formation of 12HETEs in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as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

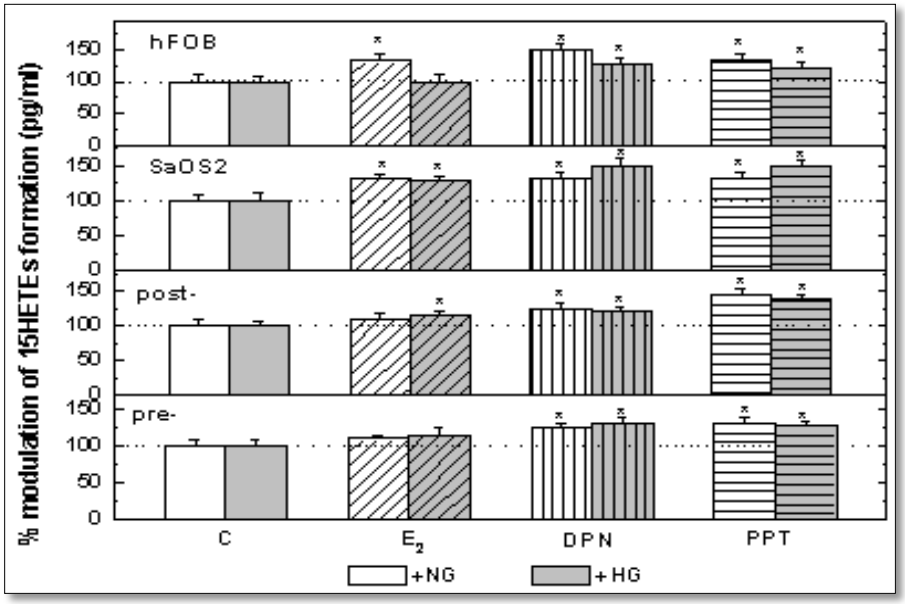


**Figure 8d.** Modulation by HG (44 nM compared to 22 nM) of the stimulation by $E_2$, DPN and PPT of the formation of 15HETEs in primary human female-derived osteoblasts as well as SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as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

**ACKNOWLEDGEMENT**

I want to thank my colleagues Katzburg S, Knoll E, Sharon O and Stern N for their help in this research study and Posner G for providing the vitamin D analogs.

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