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Hyperglycemia Modulates the Effects of Vitamin D Analogs on Human Derived Female Cultured Bone Cells and Female Bone Cell Lines

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ABSTRACT

Human female derived cultured osteoblasts at different ages (pre- and post-hObs) and human female bone cell lines (SaOS2 and hfoB) express mRNAs which are important for 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 expressions and biological activities are affected by vitamin D analogs. We now tested the modulations by vitamin D analogs 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 basal DNA synthesis (DNA) and Creatine Kinase specific activity (CK). Stimulations of DNA but not of CK by vitamin D analogs were modulated by HG.

Itself up-regulated the expression of mRNA of 12LO and 15LO and to less extent ERβ and VDR, but did not affect ERα and 1OHase mRNA expression. The treatments with the vitamin D analogs JKF and QW modulated the expression of VDR, 1OHase, 12LO and 15LO mRNAs which were reduced in HG. The induction of synthesis 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 by which HG affect bone cell responses and its relevance to human bone physiology has to be further studied.

Keywords: Human osteoblasts, Hyperglycemia, ERs, VDR, 1OHase, LOs, Vitamin D analogs

INTRODUCTION

We have previously studied the effects of estrogens on rat bone physiology using the induction of the specific activity of creatine kinase (CK) as a response marker [1]. 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 an efficient response marker to different hormones including vitamin D analogs, in the physiology of bone cells *in vivo* and *in vitro* [2], which contain detectable concentrations of vitamin D receptors (VDR) [3].

Diabetes is associated with a net loss of bone (Consensus opinion of The North American Menopause Society, 2000), resulted in the reduction of new bone formation and in the decrease of bone mineral density. In diabetic mice the up-regulation of specific transcription factors is attenuated, resulting in lower conversion of mesenchymal cells to osteoblasts [4].

We have found previously that vitamin d analogs stimulate also vitamin D (VDR) and 1OHase mRNA expression and activity as measured by 1α, 25 hydroxy vitamin D_3 (1,25D) formations [5]. Vitamin D analogs as well as other hormones induce also lipoxygenase mRNA (12 and 15LO) expression and activity which is measured by HETE formation (12 and 15HETE) [6].

In the present study we set to analyze the effects of High Glucose (HG) in the growth medium on the response to vitamin D compounds of human-derived cultured bone cells,

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as well as the human female bone cell lines namely SaSO2 and hfoB, which is relevant to the important factors existing in diabetes. The compounds analyzed were the less calcemic vitamin D analogs JKF and QW [6].

In the present study we analyzed:

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

MATERIALS AND METHODS

Reagents

All reagents used were of analytical grade. Creatine Kinase (CK) assay kit was purchased from Sigma Chemicals Co. (St. Louis, MO). JKF and QW were synthesized and obtained from Prof Gary Posner.

Cell cultures

1. Human bones were obtained from biopsies of patients undergoing surgery, after 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). 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. Briefly, 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 [7], 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 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 1 nM JKF and QW for 24 h or daily additions for 3 days, 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 [7]. 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.

DNA synthesis assessments

Cells were grown until sub-confluence and then treated with various hormones as indicated for CK. 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 ERa, ERb, VDR, 25 hydroxy vitamin D3 1-α hydroxylase (1OHase), 12 lipooxygenase (12LO) and 15LO by real time PCR

RNA was extracted from cultured human bone cells or cell lines and subjected to reverse transcription as previously described [8-10].

Assessment of 1OHase activity

1OHase activity was assessed by the measurement of 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 the culture, using 1,25D ^{125}I RIA kit from Dia Sorin, Mn, USA [11]. Protein was assayed by Coomassie Brilliant Blue dye binding, using BSA as the 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 [10].

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

HG modulation of DNA synthesis and CK specific activity induced by JKF and QW in human female-derived osteoblasts

Basal activities in the different bone cells demonstrate that hObs from pre-menopausal females show the highest activity of both DNA synthesis and CK specific activity. Growing the cells in HG increased constitutive level of the specific activity of CK in pre-menopausal hObs by 146 ± 5% and in post-menopausal hObs by 134 ± 8%, in SaOS2 by 135 ± 15% and no effect in hfoB 110 ± 8%. Growing cells in HG increased also basal level of DNA synthesis (DNA) in pre-menopausal hObs by 153 ± 20% and in post-menopausal hObs by 165 ± 13%, in SaOS2 by 200 ± 12% but no effect in hfoB by 108 ± 8%. Treatment with the Vitamin D analogs JKF and QW for 24 h, showed a significant increase in DNA in both age groups and in both cell lines **(Figure 1)**. The response of pre-menopausal cells was higher than that of post-and SaOS2 and the same as in hfoB with JKF and QW **(Figure 1)**. Growth of the cells in HG led to reduction of the response of CK to treatment with JKF and QW in all cells tested **(Figure 1)**. Growth of the cells in HG led to reduction of the response of DNA to treatment with JKF and QW in all cells tested **(Figure 2)**.

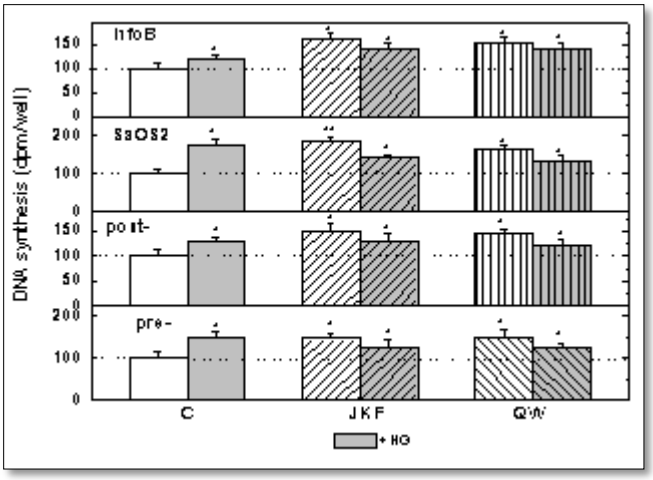

Figure 1a. Stimulation by 1nM JKF or QW of DNA synthesis in primary human female derived osteoblasts and SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and treated for 24 h with the vitamin D analogs and extracts prepared for analysis as described in Materials and 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): *, $P<0.05$; **, $P<0.01$.

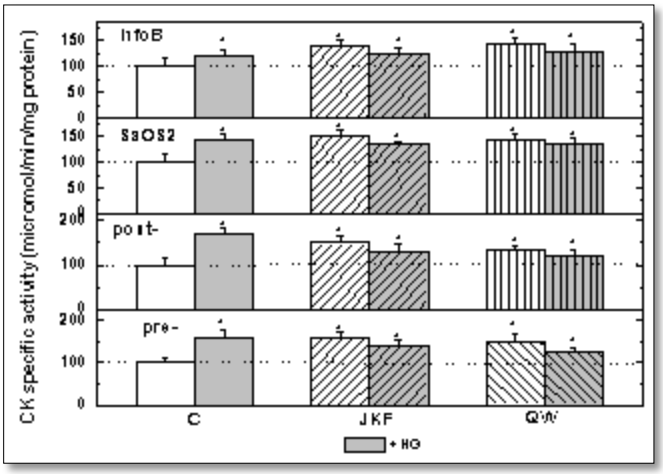

Figure 1b. Modulation by HG (44 nM compared to 22 nM) of the stimulation by 1 nM JKF or QW of CK specific activity in primary human female- derived osteoblasts and SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for analysis as described in Materials 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): *, $P<0.05$; **, $P<0.01$.

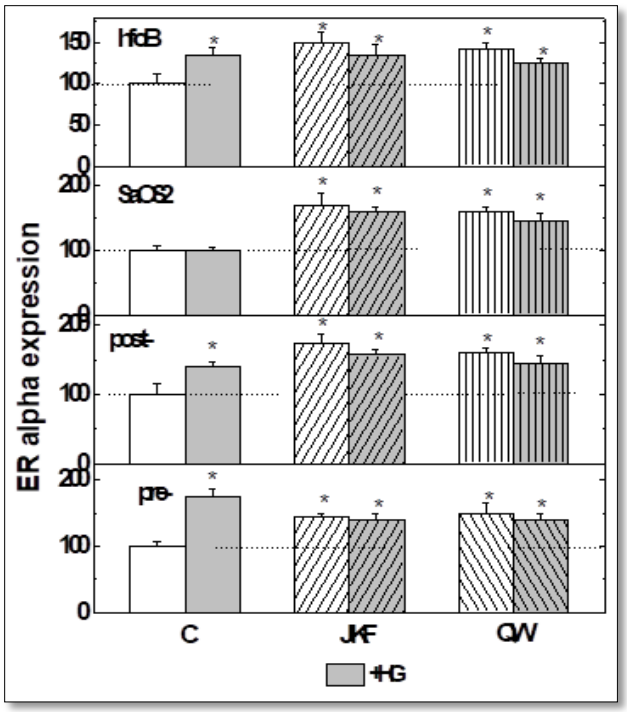

Figure 2a. Modulation by 1nM JKF or QW of the expression of mRNA for ERα in primary human female derived osteoblasts and SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for real time PCR analysis as described in Materials and Methods. Results 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.

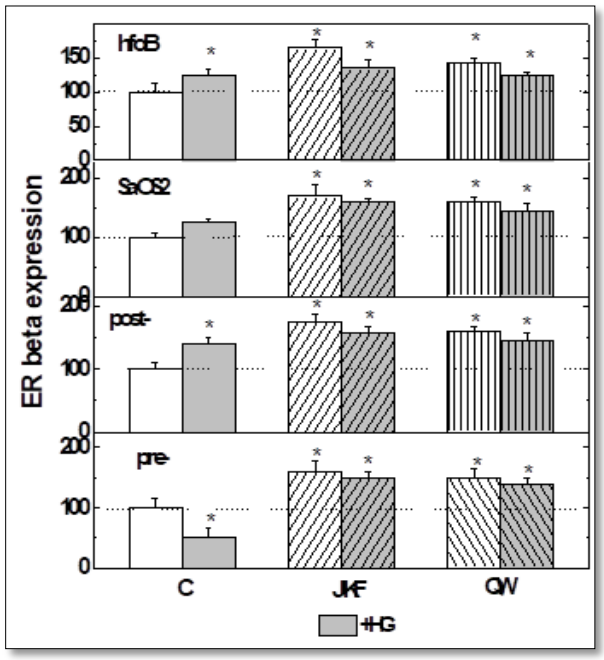

Figure 2b. Modulation by HG (44 nM compared to 22 nM) of the modulation by 1 nM JKF or QW of the expression of mRNA for ERβ in primary human female derived osteoblasts and SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for real time PCR analysis 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.

HG modulation of the expression of ERα and ERβ in human female-derived osteoblasts

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. High glucose increased the expression of both ERα and ERβ, in the different cells to different extents except in SaSO2 for ERβ. Female derived hObs treated with JKF and QW, showed a significant increase in ERα **(Figure 3a)** and ERβ **(Figure 3b)**, whereas in HG the increase was smaller **(Figure 3a and Figure 3b)**.

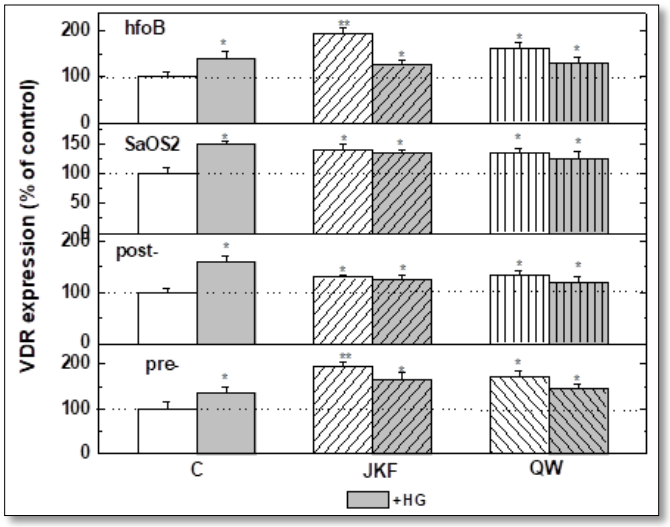

Figure 3a. Modulation by HG (44 nM compared to 22 nM) of the modulation by 1 nM JKF or QW of the expression of mRNA for VDR in primary human female-derived osteoblasts and SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for real time PCR analysis 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): *, $P<0.05$; **, $P<0.01$.

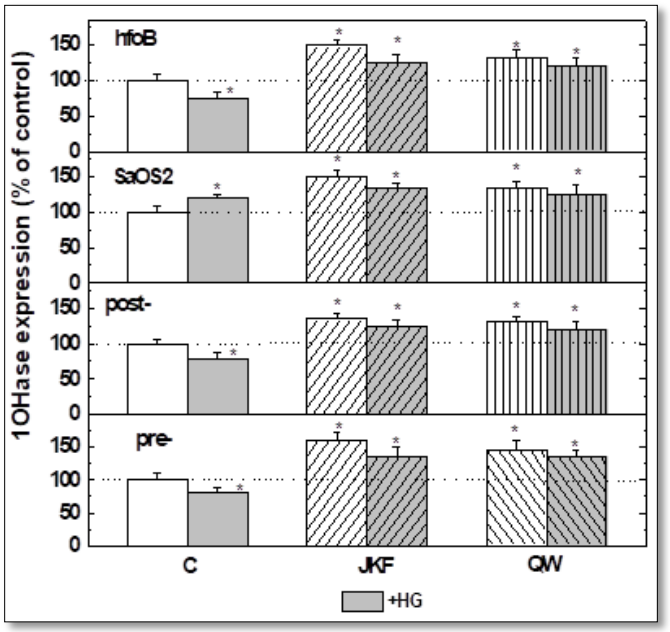

Figure 3b. Modulation by HG (44 nM compared to 22 nM) of the modulation by 1 nM JKF or QW of the expression of mRNA for 1OHase in primary human female derived osteoblasts and SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for real time PCR analysis 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): *, $P<0.05$; **, $P<0.01$.

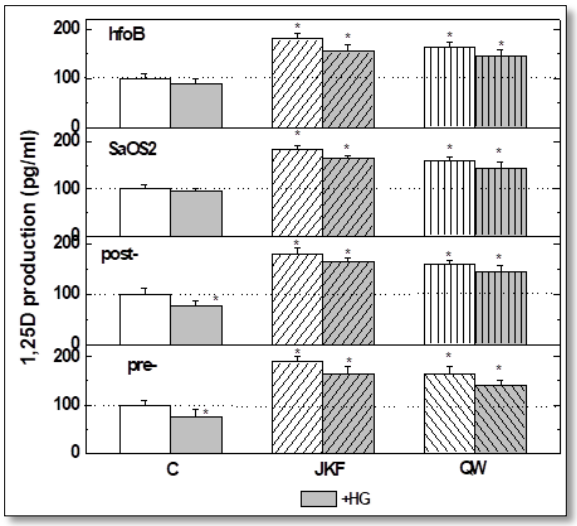

Figure 3c. Modulation by HG (44 nM compared to 22 nM) of the stimulation by 1 nM JKF or QW of the formation of 1,25D in primary human female derived osteoblasts and SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described in Materials 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): *, $P<0.05$; **, $P<0.01$.

HG modulation of the expression of VDR and 1OHase as well as 1,25D production in human female-derived osteoblasts

Female-derived osteoblasts 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 and also produced $1,25(OH)_2D_3$ (1,25D) as measured by radio-immunoassay. Growing the cells in HG decreased both the expression of 1OHase and 1,25D production in both age groups and hfoB but not in SaSO2. On the contrary growing the cells in HG increased VDR in all cells tested.

Female derived hObs treated with JKF and QW, showed a significant modulation in VDR **(Figure 4a)**. In all cells HG decreased VDR stimulated expression **(Figure 4a)**. Similar results were obtained with both 1OHase expression and its activity as measured by 1,25D production **(Figures 4b and 4c)**.

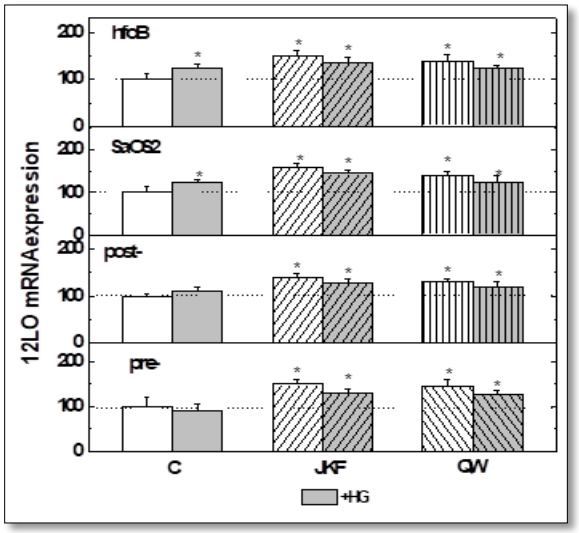

Figure 4a. Modulation by HG (44 nM compared to 22 nM) of the stimulation by 1 nM JKF or QW of the expression of mRNA for 12LO in primary human female- derived osteoblasts and SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for real time PCR analysis 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): *, $P<0.05$; **, $P<0.01$.

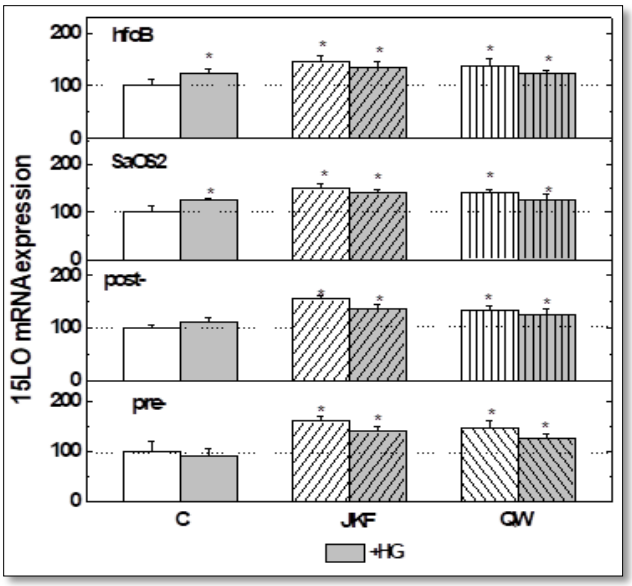

Figure 4b. Modulation by HG (44 nM compared to 22 nM) of the stimulation by 1 nM JKF or QW of the expression of mRNA for 15LO in primary human female- derived osteoblasts and SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for real time PCR analysis 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): *, $P<0.05$; **, $P<0.01$.

HG modulation and expression of 12LO and 15LO in human female-derived osteoblasts

Female-derived bone cells from both female age groups and cell lines expressed mRNA for 12LO and 15LO as measured by real-time PCR. Growing the cells in HG increased the expression of 12LO and 15LO in all cells to different extent except hfoB. Female derived hObs treated with JKF and QW increased 12LO mRNA expression, in all cells which was reduced by HG **(Figure 5b)**. Treatment with JKF and QW in NG increased the 15LO mRNA expression **(Figure 5b)**. In HG, the increase in 15LO mRNA expression by the different vitamin D analogs was slightly reduced compared to NG **(Figure 5b)**.

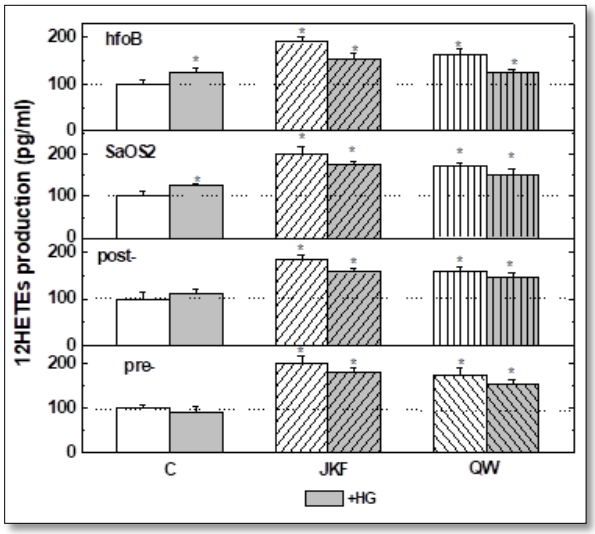

Figure 5a. Modulation by HG (44 nM compared to 22 nM) of the stimulation by 1 nM JKF or QW of the formation of 12HETEs in primary human female derived osteoblasts and SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured, and extracts prepared for analysis as described in 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): *, $P<0.05$; **, $P<0.01$.

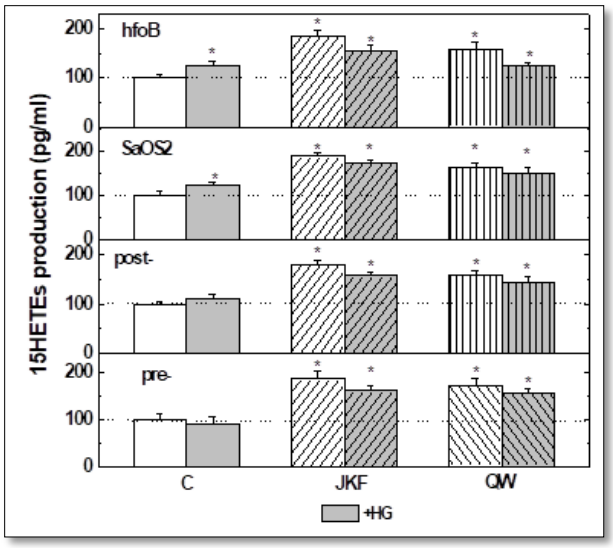

Figure 5b. Modulation by HG (44 nM compared to 22 nM) of the stimulation by 1 nM JKF or QW of the formation of 15HETEs in primary human female derived osteoblasts and SaOS2 and hfoB human female bone cell lines. Bone cells were obtained and cultured and extracts prepared for analysis as described in 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): *, $P<0.05$; **, $P<0.01$.

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

Female derived bone cells from both female age groups and cell lines produced 12HETE and 15HETE. Growing the cells in medium containing HG increased the production of 12HETE and 15HETE in all cells to different extent. Female derived hObs treated with JKF and QW increased 12HETE and 15HETE production, in all cells tested. In HG, the increase in 12HETE and 15HETE production by JKF and QW was slightly down-regulated.

DISCUSSION AND CONCLUSION

JKF and QW 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 22 mM) sharpens the differences between the different groups. First of all hyperglycemia increased the constitutive levels of DNA and of CK in all cells used except in hfoB cell line. Moreover, the stimulation of DNA and CK by JKF and QW was slightly decreased by hyperglycemia in both age groups and SaOS2 cell line but not in the hfoB cell line. It is important to note that the constitutive levels of DNA synthesis and CK specific activity were increased by HG in all age group bone cells and both cell lines.

Bone growth which is disturbed in diabetes [4,12] was also not enhanced to the same extent by hormone replacement therapy as shown before.

Bone cells express VDR and 1OHase which is the enzyme synthesizing the active of vitamin D metabolite $1,25(OH)_2D_3$ (1,25D). Hyperglycemia increased only VDR expression but decreased 1OHase expression and activity as measured by 1,25D formation. Whether these metabolic changes are leading to modulations of bone physiology as a result of the possible changes in 1,25D levels is not yet clear. The modulation of VDR and 1OHase as well as 1,25D, is additional important information to the spectrum of changes due to hyperglycemia.

Recent studies linked 15LO and 12LO (platelet type) to bone density. In the present study we show that the expression of the LOs in these cells is modulated by growing the cells in HG. 12LO mRNA is increased by HG in all cells and 15LO mRNA is also increased in those cells. Growing the cells in HG slightly reduced the induction of 12LO and 15LO mRNA by JKF and QW. The expression of these enzymes leads to the ability of bone cells to produce and secrete 12HETE and 15HETE, the products of LOs. 12HETE as well as 15HETE production is increased by growing the cells in high glucose. Growing the cells in HG decreased the induction of 12 HETE and 15HETE by JKF and QW.

The exact mechanism of the effects of growing the cells in HG in the growth medium on bone cell responses to vitamin D 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 with different drugs which might be less affected by hyperglycemia. If these proposed future experiments show promising results, we will analyze animal models which might lead to human studies in order to understand these models [13].

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