EFFECT OF EXPERIMENTAL DYSGLYCEMIA ON UNDER-CARBOXYLATED OSTEOCALCIN PRODUCTION IN HUMAN PRIMARY OSTEOBLAST-LIKE CELL CULTURES

A. Kacso^{1,**}, M. Goia-Socol^{1,**}, G. Hazi⁴, G. Tomoaia^{2,5}, I.M. Kacso^{3,6}, C.E. Georgescu^{1,7,*}

“Iuliu Hatieganu” University of Medicine and Pharmacy Cluj-Napoca, ¹Department of Endocrinology, ²Department of Ortopedics and Traumatology, ³Department of Nephrology, Cluj County Emergency Hospital - ⁴Hormonology Laboratory, ⁵Orthopedics and Traumatology Clinic, ⁶Nephrology Clinic, ⁷Endocrinology Clinic, Cluj-Napoca, Romania

Abstract

Context. The undercarboxylated form of osteocalcin (ucOC) and osteoprotegerin (OPG) are bone-derived molecules involved in the endocrine crosstalk governing the bone, the adipose tissue and the pancreas. In addition, glucocorticoids are major determinants of both insulin resistance and osteoporosis.

Objective. We aimed to investigate the response of ucOC and OPG to dysglycemia and/or dexamethasone (DXM) in primary human osteoblastic cell (HOC) cultures.

Design and methods. Third-passage sub-confluent primary HOC cultures were treated with glucose: 2.8 mmol/L, 5.6 mmol/L, 11.1 mmol/L and 28 mmol/L, respectively. Alternatively, HOC cultures were subjected to DXM 1 µmol/L. In more complex experiments, HOC cultures were pre-treated with glucose (5.6 mmol/L) with/without insulin (1 pmol/L) followed by DXM (1 µmol/L). 24-hours post-treatment, culture medium ucOC and OPG were measured by ELISA.

Results. ucOC production differed significantly ($p < 0.05$) between cell groups, decreasing in a dose-dependent manner as glucose concentration in the medium increased. Insulin prevented this effect. OPG levels appeared not to be significantly influenced by the hyperglycemic culture medium and were not related to ucOC concentration ($p > 0.05$). Addition of DXM resulted in significantly lower ucOC concentrations compared to vehicle-treated cells ($p < 0.05$). However, the effect of insulin co-treatment on ucOC was not counteracted by DXM ($p < 0.05$).

Conclusions. An obvious alteration of OC production/metabolism was observed as glucose levels changed in the bone microenvironment, to potentially be involved in diabetes-related osteopenia. DXM suppressed ucOC levels however not in insulin-rich environment.

Key words: human osteoblasts, undercarboxylated osteocalcin, osteoprotegerin, insulin, dexamethasone, osteopenia, diabetes mellitus.

INTRODUCTION

The recently acknowledged endocrine crosstalk governing the bone, the adipose tissue and the pancreas hints towards a complex regulation of energy metabolism. One of the key molecules of this system is osteocalcin (OC, also known as bone γ -carboxyglutamic acid (Gla) protein), the most abundant non-collagenic protein of bone (1). Osteocalcin belongs to the vitamin K dependent protein family, vitamin K being a cofactor for its γ -carboxylation, which determines its high affinity for bone matrix and calcium, thus contributing to bone formation (2). However, it is the undercarboxylated form of osteocalcin (ucOC) that exerts hormonal properties and is involved in glucose and energy metabolism (3). In brief, osteoblastic ucOC stimulates insulin secretion in the pancreas, both directly and indirectly by enhancing glucagon-like-peptide 1 (GLP-1) synthesis from the small intestine and promotes insulin sensitivity in adipocytes and myocytes (4, 5). Conversely, patients with insulin resistance and diabetes may present with low OC levels (6), a feature which may affect the bone mineralization process, this further potentially altering bone quality (7) and increasing fracture risk (2). Interference of metabolic pathways with bone metabolism may potentially affect bone mass (8, 9).

A second bone molecule with possible metabolic implications is osteoprotegerin (OPG), a protector against bone resorption. While OPG may be involved in diabetes vascular calcifications, contradictory results were reported regarding its levels in patients with type 2 diabetes mellitus (5).

Glucocorticoids, largely used nowadays for anti-inflammatory purposes, represent another link

*Correspondence to: Carmen Emanuela Georgescu MD PHD, “Iuliu Hatieganu” University of Medicine and Pharmacy Cluj-Napoca, Discipline of Endocrinology, 6th Medical Specialties Dept, 44F Eugen Ionesco St, Cluj-Napoca, 400496, Romania, E-mail: c_e_georgescu@yahoo.com

**These authors share the same contribution.

between bone, adipose tissue and the pancreas, as they are major determinants of both insulin resistance and osteoporosis. Osteocalcin was found as a possible mediator of their metabolic activities (10). Notably, detrimental effects of glucocorticoids on bone metabolism have been extensively demonstrated both *in vitro* and *in vivo* (11).

We therefore aimed to investigate the complex connections in primary human osteoblastic cell (HOC) cultures treated with glucose, insulin and dexamethasone (DXM) by assessing OC in its undercarboxylated form and OPG, in order to characterize their response to dysglycemia and/or glucocorticoid-rich microenvironment.

MATERIALS AND METHODS

Approval to perform the experiments was obtained from the “Iuliu Hatieganu” University Academic Ethics Committee.

Materials

Glucose (D-(+)-Glucose), insulin (Insulin, human), dimethylsulfoxide (DMSO) and dexamethasone (DXM) were all purchased from Sigma-Aldrich Chemie (Germany). Dulbecco's Modified Eagle Medium (DMEM) and penicillin G/streptomycin/glutamine were used to culture osteoblasts and obtained from Invitrogen Life Technologies (USA). Fetal bovine serum (FBS) was purchased from Sigma-Aldrich Chemie (Germany). Commercial kits were used to assay ucOC (Takara Bio Inc., Otsu, Japan) and OPG (Biomedica Medizinprodukte GmbH & Co, Vienna, Austria), respectively.

Primary human osteoblastic cell (HOC) cultures

The cell culture method followed the protocol described by Pepene *et al.* 2001 (12) and Goia-Socol M *et al.* (7). Human bone cells were obtained from donors subjected to hip arthroplasty during surgery. None of the patients suffered from diabetes or any other endocrine or metabolic disorder or was on bone-active medication. Bone fragments were dissected, fragmented and seeded into 9-cm diameter plastic culture dishes, further cultured under standard conditions, specifically in phenol-red DMEM supplemented with 10% FBS and 1% penicillin G/streptomycin/glutamine at 37°C temperature and 5% CO₂ atmosphere. Twice weekly the culture medium was exchanged for fresh medium. To perform the experiments we used third-passage

sub-confluent cell cultures. Twenty-four hours prior to further experiments, the culture medium was replaced by phenol red-free DMEM supplemented with 10% FCS and 1% penicillin G/streptomycin. HOC cultures were treated with various concentrations of glucose, specifically 2.8 mmol/L, 5.6 mmol/L, 11.1 mmol/L and 28 mmol/L, respectively, for 24h, after which supernatants were collected. Alternatively, HOC cultures were subjected to DXM 1 µmol/L for 24h. In more complex experiments, HOC cultures were pre-treated for 24h with glucose (5.6 mmol/L) with or without insulin (1 pmol/L) followed by DXM (1 µmol/L) for 24h. DMSO and ethanol were used as controls, accordingly. Experiments were performed in triplicates.

Bone matrix proteins assays

Culture medium ucOC was measured by a commercial two monoclonal antibodies ELISA. The mean intra-assay CV was 4.4%. OPG secreted by osteoblasts in the culture medium was determined using a commercial ELISA kit with intra-assay CV between 4 and 10%.

Statistical analysis

Data were expressed as mean ± SD. Differences between groups regarding ucOC or OPG secretion into culture medium were analyzed using Student's t-test or Mann-Whitney test. To compare several groups, analysis of variance (ANOVA) was performed. Pearson's correlation coefficient was calculated to indicate the association of ucOC and OPG levels.

RESULTS

Osteoblastic cell cultures subjected to dysglycemia were reexamined after 24 hours when the culture medium was harvested. A significant difference was observed in ucOC production between various cell groups ($p < 0.05$, by ANOVA) thus suggesting an obvious alteration of OC synthesis and/or metabolism as glucose levels change in the bone microenvironment. In fact, as the glucose concentration in the medium increased, ucOC production decreased in a dose-dependent manner (Fig. 1).

Likewise, primary HOC cultures treatment with DXM 1 µmol/L resulted in significantly lower ucOC concentrations compared to vehicle-treated cells ($p < 0.05$, Fig. 2), to emphasize that glucocorticoids might directly impact on OC production in the bone. In contrast, OPG levels appeared not to be significantly

influenced by even severe hyperglycemic bone microenvironment. Moreover, at 24h, OPG release into medium failed to be related to ucOC concentration ($p>0.05$, by Pearson's correlation, Fig. 3), thus, suggesting other determinants could be involved as more important predictors of ucOC generation in bone cells *in vitro*.

Adding insulin 1 pmol/L to balance the hyperglycemic culture medium (~100 mg/dL) prevented the deleterious effect of hyperglycemia on ucOC, which significantly increased compared to that assayed in cultures further maintained under hyperglycemic conditions ($p<0.05$, Fig. 4). Furthermore, the effect of insulin on ucOC production was not counteracted by DXM ($p<0.05$ by ANOVA).

DISCUSSION

Both OC and OPG promote bone mass acquisition but are also involved in energy metabolism and our results are coming to enlarge this complex picture.

The first part of our experiments aimed to assess the influence of various glucose concentrations in the HOC environment on osteoblastic OC and/or OPG production. We found that ucOC production decreased in a dose-dependent manner as the glucose concentration in the medium increased. This effect was acknowledged at a concentration of 5.6-mmol/L glucose in the culture medium, which resembles to normal glucose microenvironment. Similar results

Figure 1. Inhibition of undercarboxylated osteocalcin (ucOC) production by glucose in primary human osteoblast-like cultures (HOC). Osteoblast-like cultures were subjected to various concentrations of glucose, specifically 2.8, 5.6, 11.1 and 28 mmol/L for about 24 hours. Ethanol was used as control. G = glucose; Eth = Ethanol; * $p<0.05$.

Figure 2. Effect of dexamethasone (DXM) 1 μ mol/L (24h) on undercarboxylated osteocalcin (ucOC) delivery in culture medium of primary human osteoblast-like cultures (HOC) * $p<0.05$.

Figure 3. Inhibition of osteoprotegerin (OPG) production by glucose in primary human osteoblast-like cultures (HOC). Osteoblast-like cultures were subjected to various concentrations of glucose, specifically 2.8, 5.6, 11.1 and 28 mmol/L for about 24 hours. Ethanol was used as control. G = glucose; Eth = Ethanol; * $p<0.05$.

Figure 4. Effect of insulin or insulin and dexamethasone (DXM) on undercarboxylated osteocalcin (ucOC) secretion in primary human osteoblast-like cultures (HOC) during experimental dysglycemic conditions. INS (1pmol/L) prevented inhibition of glucose (5.6mmol/L)-dependent ucOC secretion by HOC. The effect is not counteracted by dexamethasone (DXM). $p<0.05$ (ANOVA). G = glucose

with ours were obtained by a group of researchers who evaluated osteoblasts at 72 hours stimulation with 5.5 and 22 mM glucose microenvironment, respectively (13). Nevertheless, another study assessing OC mRNA levels at 12 and 24 mM glucose concentration in the culture environment obtained opposite results. It should be taken into account, however, that this study evaluated osteoblasts after 7 and 14 days of culture in the hyperglycemic medium, whereas in our study the evaluation was done after 24h (14). Furthermore, those results still need confirmation as at 7 culture days high glucose levels (49.5 mM) appeared to inhibit osteoblastic growth (15); on top of that, chronic hyperglycemia (30 mM) was associated with lower osteocalcin in 14, 21 and 29 days of osteoblastic cultures (15).

Furthermore, our observations were consistent to those revealed by clinical studies on the inhibitory effect of high glucose on ucOC. Indeed, low ucOC levels were detected in diabetic and insulin resistant patients' serum, which correlated inversely to HbA1c (1).

A second purpose was to examine the effect of insulin on ucOC and OPG. We noticed that insulin was able to prevent ucOC reduction previously observed under hyperglycemic conditions, and the result was statistically significant. A similar outcome was present also in the study of Wu YY *et al.* (13). It appears that not only glucose and insulin by themselves have such significant effects on osteoblastic ucOC secretion, but also insulin signaling in osteoblasts, which is mediated by the insulin receptor (IR). High glucose appears to decrease IR gene expression, while the combined treatment with glucose and insulin amplifies its expression, thus, suggesting that the presence of insulin augments its signaling in osteoblasts (12). This *in vitro* model was confirmed in clinical studies, where, as mentioned above, serum ucOC was found to be low in insulin resistant patients (1).

OPG levels appeared not to be significantly influenced by glucose concentrations in the culture medium in our study. Moreover, at 24h, OPG release into medium failed to be related to ucOC concentration. Other researchers found OPG decreased in high glucose medium, however, this was encountered at 7 and 14 days of culture (13). On the other hand, in a meta-analysis, OPG was found to be significantly elevated in diabetic patients compared to controls (16). Moreover, OPG was higher in patients with type 2 diabetes treated with oral antihyperglycemics compared with type 1 (17). These differences may suggest that the presence or absence of insulin in type 1 diabetes on the one hand or the impact of insulin resistance or insulin treatment

in type 2 diabetes on the other hand may be responsible of the variation in OPG levels. Other contributors appear to be the anti-hyperglycemic medication used, the differences in BMI or duration of diabetes. However, addition of insulin to the culture medium in our HOC experiment led to no additional effect on OPG secretion. Such heterogenic results between various studies suggest this topic needs further research.

Going to the third part of our study, glucocorticoid influence on ucOC and OPG was assessed. Primary HOC cultures treatment with DXM 1 $\mu\text{mol/L}$ resulted in significantly lower ucOC concentrations compared to vehicle-treated cells thus suggesting that glucocorticoids might directly influence the production/carboxylation of OC within the skeleton. It was previously confirmed, in animal models, that the disruption of glucocorticoid signaling significantly decreased the suppression of OC synthesis and prevented the development of insulin resistance, glucose intolerance, and abnormal weight gain in glucocorticoids-treated mice (18). However, the effect of insulin on ucOC production by HOC cultured in hyperglycemic medium was not counteracted by DXM in the concentration used in our research.

Overall, the present study contributes to the new-emerging complex picture regarding the regulation of energy metabolism, which explains the stress - body weight - bone mineral density correlations present in our daily medical practice (10,19).

In conclusion, obvious alterations of OC production and/or metabolism were observed as glucose levels changed in the bone microenvironment supporting the already known relationships between OC and energy metabolism. Moreover, they may point towards a role of unbalanced OC production (possibly to reflect bone formation inhibition) and/or metabolism in the mechanisms of diabetes-induced low bone mass. However, OPG release into the medium failed to be related to ucOC concentration, suggesting other determinants are more important predictors of ucOC generation in bone cells *in vitro*. DXM suppressed ucOC levels but not in insulin-rich environment. These last observations would benefit of further research towards proper metabolic mechanisms elucidation.

Conflict of interest

The authors declare that they have no conflict of interest.

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