ORIGINAL RESEARCH

The Effect of Oral Glucose Tolerance Test on Serum Osteocalcin and Bone Turnover Markers in Young Adults

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Abstract Osteocalcin (OC) is an osteoblast-derived protein implicated in the regulation of glucose tolerance and energy metabolism. This endocrine function has been suggested to be exerted via its undercarboxylated form, which has been shown to induce expression of adiponectin, insulin, and islet cell proliferation in mice. Furthermore, insulin has recently been shown to regulate the biological activity of OC in bone. Our aim was to explore the association between glucose and bone metabolism by evaluating the effect of a standard 75 g oral glucose tolerance test (OGTT) on serum OC, carboxylated OC (cOC) and bone-turnover markers (BTMs) C terminal telopeptide (βCTX-I) and N terminal propeptide (PINP) of type I collagen and tartrate-resistant acid phosphatase 5b (TRACP5b). Serum samples collected at 0 and at 120 min were analyzed in a cohort of normoglycemic young adults ($n = 23$, mean age 23.6 years). During OGTT a significant decrease was observed in all BTMs ($P < 0.001$ for all variables). The median decreases from 0 to 120 min for OC, cOC, βCTX-I, PINP, and TRACP5b were -32.1% (-37.9 to -19.6), -34.4% (-39.8 to -22.2), -61.4% (-68.5 to -53.0), -26.8% (-33.2 to -19.2), and -44.5% (-48.3 to -40.2), respectively. A strong association between the changes in OC and cOC was observed ($r = 0.83$, $P < 0.001$). The decrease in PINP was associated with changes in OC, whereas the changes in βCTX-I and TRACP5b were not associated with decreases in OC or cOC. The observed OGTT-induced changes in bone-derived proteins were partially independent of each other and potentially mediated by different mechanisms.

Keywords Serum osteocalcin · Bone turnover marker · Oral glucose tolerance test

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Osteocalcin (OC) is an osteoblast-derived protein which is secreted into the circulation during bone formation. Serum OC (S-OC) has widely been used as an established biomarker of bone turnover and bone formation [1], but numerous studies have also evaluated the potential role of OC as a predictor and surrogate marker of fracture risk or osteoporosis [2–4]. Recently, OC has also been recognized as an endocrine factor: it plays a role in the regulation of glucose tolerance and energy metabolism in mice by influencing insulin secretion and insulin sensitivity [5, 6]. This endocrine function of OC is most likely regulated via vitamin K-dependent posttranslational modification, γ-carboxylation, as only the uncarboxylated form of OC has been shown to induce expression of adiponectin, insulin, and markers of pancreatic islet cell proliferation [5, 6]. Until recently the degree of γ-carboxylation has mainly been considered as a marker of vitamin K status in bone [7], but an increasing body of evidence indicates that

biological activity of OC is linked to uncarboxylated OC and to the degree of carboxylation of OC [5, 6, 8].

Only a limited number of studies have evaluated the complex interactions between the skeleton and glucose and energy homeostasis in humans. An inverse association between OC and markers of metabolic dysfunction, such as hyperglycemia, insulin resistance, and glucose intolerance, has been reported in clinical studies [9, 10]. While the reported associations are relatively strong, no direct causality between changes in fasting glucose, insulin sensitivity, and changes in OC or its degree of carboxylation have been confirmed. Interpretation of the results is complicated as these studies are seldom prospective and include mostly cross-sectional post hoc analyses of elderly populations with confounding factors related to high age and comorbidities such as type 2 diabetes, impaired glucose tolerance, and dyslipidemia [9–14].

Recently, a feed-forward regulatory loop was identified when insulin was shown to influence osteoblasts, activate bone remodeling, and regulate the biological activity of OC in the bone microenvironment [8]. This novel regulatory loop links insulin, bone cells, and OC into a two-directional regulatory pathway in which OC regulates insulin and insulin influences OC [15]. In order to evaluate if the same principles apply to human physiology, it would be important to study if concentrations of OC change in response to glucose load and subsequent eating-promoted insulin release. Whereas bone resorption has been shown to decrease postprandially in humans [16, 17], the effects of a meal or nutrient absorption on bone formation and OC have only been studied in subjects with multiple confounding factors, generating mixed results [17–19]. None of the previous studies have evaluated the effect of rapidly applied glucose load on the regulation of bone homeostasis and whether it induces a biologically significant and measurable effect on levels of serum total OC (S-TotalOC) and carboxylated OC (cOC) in normoglycemic adults close to the age of their peak bone mass.

Our aim was to explore the acute glucose-induced changes in bone metabolism, particularly on OC, in a cohort of young adults.

Materials and Methods

Subjects

This study included 23 healthy subjects (12 females, 11 males) aged 18–26 years who were invited as a pilot group to participate in a study assessing various adult health-related parameters, including glucose tolerance [20]. The Helsinki University Central Hospital Ethics Committee approved the study protocol, and written informed consent was given by all study participants. The study was carried out according to the principles of the Declaration of Helsinki.

Oral Glucose Tolerance Test

All subjects attended the clinic at the National Public Health Institute after an overnight fast of at least 10 hours. Subjects were evaluated for clinical characteristics, medical history (including their parents' history of type 1 and type 2 diabetes), and markers of adiposity and glucose metabolism. A 2 hour OGTT was performed between 8:05 and 11:02 a.m. after an overnight fast. Subjects ingested 75 g glucose solution, and plasma glucose and serum insulin concentrations were measured at baseline (0) and at 120 min. Impaired glucose tolerance was defined according to the World Health Organization as plasma glucose concentration <7.0 mmol/l at 0 min and/or ≥ 7.8 mmol/l but ≤ 11.1 mmol/l at 120 min. Diabetes was defined as plasma glucose concentration ≥ 7.0 mmol/l at 0 min and/or ≥ 11.1 mmol/l at 120 min. Serum samples were stored at $-70°C$ until further analysis of bone-turnover markers (BTMs).

S-OC

S-TotalOC and S-cOC were determined at 0 and 120 min by previously described protocols [21]. Two-site immunoassay of S-TotalOC was based on monoclonal antibodies (Mabs) 2H9 and 6F9 and detected the N terminal midsegment of the OC molecule. S-cOC assay (Mabs 6F9 and 3H8) detected the same fragments but preferred γ-carboxyglutamic acid (Gla)-containing forms of OC [22]. The Mabs were biotinylated and europium-labeled as described previously [22], and synthetic peptide of human OC amino acids 1–49 (Advanced Chemtech, Louisville, KY) was used as a calibrator. Samples from subjects, control samples from one donor, and calibrators (10 μl of each) were added as doublets into wells of streptavidin-coated microtiter plates (Kaivogen, Turku, Finland), followed by a mixture of biotinylated and Eu-labeled Mabs in 50 μl of Delfia® Assay Buffer (Perkin Elmer Life Sciences/Wallac, Turku, Finland) containing 5 mmol/l EDTA. For all assays, 200 ng/well of capture Mab (bio-2H9 or bio-6F9) was used and 100 ng/well of the appropriate tracer Mab (Eu-6F9 or Eu-3H8) was used. After incubation with continuous shaking at room temperature (22°C) for 2 hours, plates were washed six times with Delfia Wash Solution, and 200 μl of Delfia enhancement solution was added to each well. After 30 min shaking, time-resolved fluorescence was measured using a Victor2 Multilabel Counter (Perkin Elmer Life Sciences/Wallac).

All samples were measured simultaneously at the end of the study. In order to reduce interassay variability, samples for both time points for each subject were analyzed in parallel (baseline and 120 min samples) and in doublets. The intra- and interassay variations were 4.4 and 9.0% for S-TotalOC and 2.5 and 8.0% for S-cOC, respectively. Differences in the slope of the standard curves for total OC and cOC caused overlapping of the baseline values of OC and cOC.

Serum βCTX-I, TRACP5b, and PINP

Bone resorption was assessed by measuring serum levels of *C* terminal telopeptide of type I collagen (βCTX-I) and tartrate-resistant acid phosphatase 5b (TRACP5b) using Serum CrossLaps® ELISA and BoneTRAP® Assay, respectively (both from IDS, Tyne and Wear, UK). Bone formation was assessed by measuring serum intact *N* terminal propeptide (PINP) using the IDS-iSYS Intact PINP assay (IDS). Samples were analyzed blinded and as duplicates, except for PINP which was analyzed using a single sample. According to the manufacturer, intra- and interassay variations for the assays are for βCTX-I <6 and <10%, for TRACP5b <6 and <6%, and for PINP <4 and <6%, respectively.

Statistical Analysis

Statistical analysis was performed using SPSS, version 17.0 (SPSS, Inc., Chicago, IL). Significance was set at $P < 0.05$. The Shapiro–Wilk test was used to test for normality, and nonparametric tests were used to determine associations between BTMs as they were not normally distributed (Shapiro–Wilk test <0.95). The results of BTMs were used after logarithmic transformation. A paired *t* test was used to detect longitudinal changes during OGTT in each variable. Spearman's correlation was used to study the associations between OGTT-induced changes. Linear regression was used to study associations between different bone markers and between markers and sampling time.

Results

The baseline characteristics of our cohort of young Finnish adults ($n = 23$) are presented in Table 1. The cohort consisted of 12 females and 11 males with a mean age of 23.6 years (range 18.5–26.4). All subjects were normoglycemic at baseline (mean fasting blood glucose ± SE 4.44 ± 0.09 mmol/l and mean fasting insulin 6.5 ± 0.60 mU/l), and none had diabetes based on OGTT results. One subject with normal baseline glucose and insulin values had increased glucose (9.2 mmol/l) and insulin

Table 1 Demographics and plasma glucose and serum insulin values of the 23 study subjects

	n	Mean	Minimum	Maximum
Age (years)	23	23.6	18.5	26.4
Weight (kg)				
Females	12	58.7	42.2	76.2
Males	11	71.5	55.0	92.0
Height (cm)				
Females	12	160.2	146.6	171.5
Males	11	182.2	171.5	187.0
BMI (kg/m^2)				
Females	12	23.0	17.93	33.13
Males	11	19.1	16.51	27.29
P-Glucose (mmol/l)				
0 min	22	4.4	3.8	5.5
120 min	22	5.1	2.9	9.2
S-Insulin (mU/l)				
0 min	23	6.5	2.4	16.1
120 min	22	44.3	10.4	288.0
Baseline sampling time (a.m.)	23	9:40	8:05	11:02

(288.0 mU/l) values at 120 min, indicating signs of impaired glucose tolerance and driving the mean values for these parameters in the entire cohort. At repeated testing both the 120 min glucose and insulin values were within normal limits.

The mean absolute baseline concentrations (± SE) of S-OC and cOC were 12.8 ng/ml (±1.1) and 12.8 ng/ml (±1.2), respectively. The OGTT resulted in a statistically significant decrease from baseline to 120 min in all evaluated BTMs (paired *t* test, $P < 0.001$ for all variables) (Fig. 1). The median (interquartile range [IQR]) decreases for S-OC and S-cOC were −32.1% (−37.9 to −19.6) and −34.4% (−39.8 to −22.2), respectively, whereas reductions for the resorption markers βCTX-I and TRACP5b and the bone-formation marker PINP were −61.4% (−68.5 to −53.0), −44.5% (−48.3 to −40.2), and −26.8% (−33.2 to −19.2), respectively (Fig. 2).

The observed OC levels were independent of the time of collection. Standardized regression coefficients for OC levels and sampling time (between 8:05 and 11:02 a.m.) were for S-TotalOC 0.33 ($P = 0.128$) and for S-cOC 0.40 ($P = 0.06$).

In general, there was a strong association between the changes in S-OC and cOC (Spearman's correlation $r = 0.83$, $P < 0.001$). The decrease in the bone-formation marker PINP was highly positively associated with changes in both S-OC ($r = 0.76$, $P < 0.001$) and serum cOC ($r = 0.79$, $P < 0.001$), whereas the changes in the resorption markers βCTX-I and TRACP5b were not associated with changes in S-OC or serum cOC ($r = 0.22$, $P = 0.31$
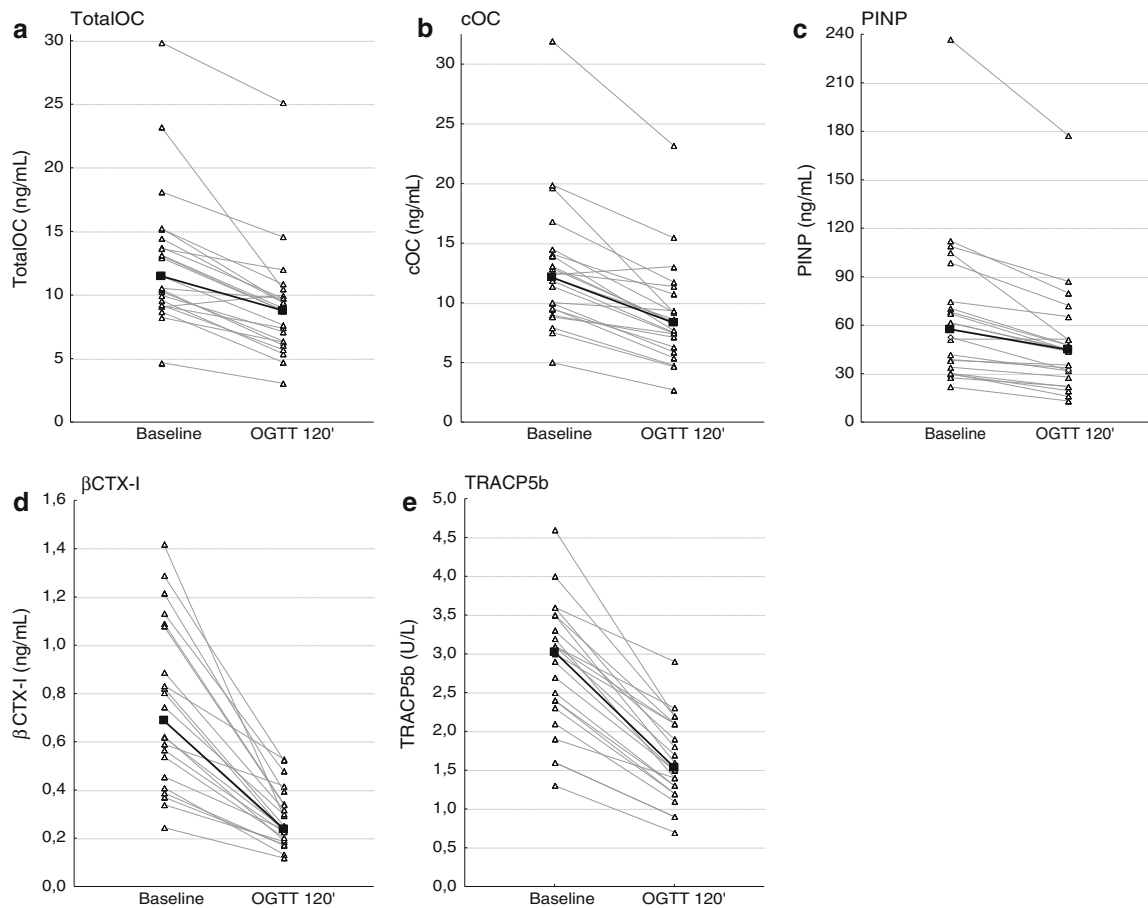
Fig. 1 BTMs before and after 120 min OGTT. Results for each individual (*open triangles*, $n = 23$) and for the median value (*solid squares*) are shown for **a** TotalOC, **b** cOC, **c** PINP, **d** βCTX-I, and **e** TRACP5b

and $r = 0.26$, $P = 0.24$, respectively). The change in βCTX-I was moderately associated with the reduction in PINP ($r = 0.42$, $P = 0.047$) and TRACP5b levels ($r = 0.44$, $P = 0.037$) (Table 2). None of the OGTT-induced changes in bone markers were associated with OGTT-induced changes in insulin or in glucose ($P > 0.05$ for all).

Discussion

Our study indicates that an acute glucose load induces a significant rapid decrease in S-OC, its carboxylated form, and markers of bone resorption (βCTX-I, TRACP5b) and formation (PINP) in normoglycemic subjects with normal insulin response during OGTT. The observed OGTT-induced decreases in these different bone-derived proteins are at least partially independent of each other. Whether these acute changes are directly induced by insulin, are general effects of glucose on bone turnover, or are mediated by OC or undercarboxylated OC remains unclear.

Acute suppression of bone turnover after intake of nutrients has been reported, and diet-induced, non-insulin-

mediated changes are the greatest in serum βCTX-I levels [19, 23] and of smaller magnitude [23–25] or not statistically significant [26] in S-OC. Feeding has been reported to have very little effect on serum TRACP5b when it was analyzed in the fed and fasting states [27]. In our study, OGTT-induced suppression in βCTX-I was $\sim 60\%$ and similar to the effect reported previously by others as a result of food intake [16, 19, 23] and oral glucose load [23–25]. Interestingly, OGTT induced also a significant reduction (median -44%) in serum TRACP5b levels. Oral glucose load induced a smaller ($\sim 30\%$) but highly significant reduction in PINP and OC. Changes observed in PINP and OC were highly correlated to each other but appeared to be independent of the suppression of bone resorption as change in βCTX-I was only moderately associated with the reduction in PINP and not associated with the changes in OC and no associations were observed between changes in TRACP5b and formation markers.

In in vitro and in vivo models insulin signaling in osteoblasts has been suggested to stimulate osteoblast differentiation and OC production, to regulate the degree of carboxylation and hence the biological activity of OC, and
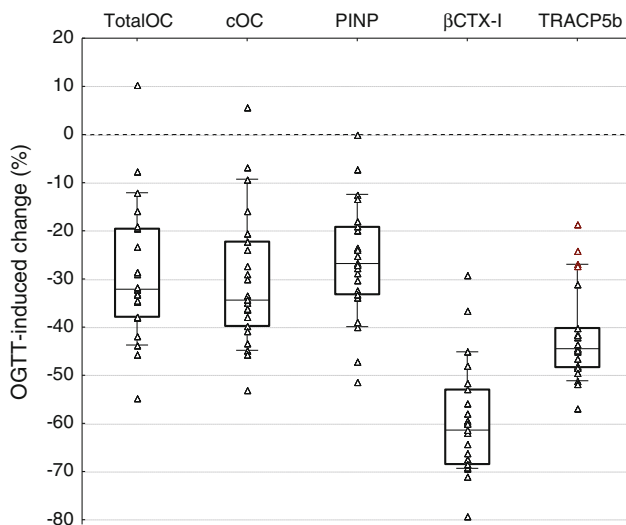
Fig. 2 Changes in BTMs TotalOC, cOC, PINP, βCTX-I, and TRACP5b induced by 120 min OGTT. Results are shown as percent change from baseline value. *Lines inside boxes* represent the 50th percentile, *limits of boxes* represent the 25th and 75th percentiles, and *whiskers* represent the 10th and 90th percentiles

Table 2 Spearman correlation (r) between OGTT-induced changes in bone markers

	cOC	PINP	βCTX-I	TRACP5b
TotalOC				
r	0.83	0.76	0.22	0.35
P	<0.001	<0.001	0.31	0.10
cOC				
r	–	0.79	0.26	0.15
P		<0.001	0.24	0.49
PINP				
r		–	0.42	0.27
P			0.047	0.21
CTX-I				
r			–	0.44
P				0.037

to upregulate bone resorption and the release of OC from the bone matrix [8, 28]. Whether a similar regulatory system is present in humans is unknown. It is also unclear how rapid this effect is and whether clinically relevant changes in markers of bone homeostasis can be observed during an OGTT-induced physiological insulin release. Small clamp studies isolating the effect of insulin on BTMs have suggested that acute changes in insulin levels do not regulate bone metabolism or carboxylation of OC [29].

Other insulinotropic peptides, such as GLP-1, GLP-2, and GIP, have been studied as potential mediators of the reductions in BTMs such as s-βCTX-I. These incretins are released from the duodenal K cells in response to carbohydrate intake [30] and are induced by an OGTT [31]. Previous studies have shown no direct effect of GLP-1 or GIP on S-OC after ingestion of macronutrients or after exogenous parenteral administration of incretins [19, 32]. Only the bone resorption marker s-βCTX-I was reduced as a response to a macronutrient meal or to exogenous GLP-2 infusion. These studies exclude both GIP and GLP-1 as key mediators for the immediate reduction in bone resorption after a meal [32]. Thus, the OGTT-induced suppression of S-OC and cOC observed in the present study is likely to be mediated via a different mechanism. It is also unclear if the mechanisms and effects of OGTT and ingestion of macronutrients on bone can be directly compared because regulation of glucose metabolism under rapidly applied, nonphysiological glucose load might be induced by different mechanisms compared to more physiological intake of nutrients. As OGTT induces a significant change in the key markers of bone turnover, the results of this study also confirm the importance of measuring these markers in the fasting state for reliable results.

The sample size in the present study was small and included only two points of measurement during OGTT; thus, no conclusions can be drawn regarding the mechanisms behind the observed OGTT-induced changes in these different bone-derived proteins. The baseline values of S-OC and cOC were also overlapping due to differences in the slope of the standard curves assessing the absolute changes in both total OC and cOC. This prohibited direct estimation of the absolute or proportional changes in the levels of uncarboxylated OC during OGTT. Furthermore, it also limited our possibilities to assess whether the parallel changes in cOC with OC merely reflected the changes in the rate of bone formation. The strengths of this study include the homogeneity of the cohort with young age, normoglycemic status, and a very limited number of potential confounding factors related to comorbidities or medications that may influence glycemia or bone turnover. The subjects were also studied close to the age of their peak bone mass.

In conclusion, we found significant decreases in serum concentrations of both total OC and its carboxylated form during an acute glucose load. Oral intake of glucose resulted also in rapid suppression of bone resorption and formation markers, indicating a general strong effect of glucose on bone turnover. Energy metabolism clearly influences bone turnover, but the direct role of insulin as the mediator of these changes needs further investigations.

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