Are There Sex Differences in the Reaction of Undercarboxylated Osteocalcin to Hypoglycemia?

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Summary

There has been increasing evidence in recent years for the hypothesis of bones as endocrine organs. Osteocalcin, long considered just a marker of new bone formation, is now seen as the first hormone produced by bones, and seems to be associated with regulating glucose metabolism and reproduction. The aim of this work was to monitor changes of osteocalcin in reaction to hypoglycemia, and determine if there are differences in such reactions between the sexes. The study included 61 healthy probands with physiological calciophosphate metabolism (30 men and 31 women). We applied to each of them an insulin tolerance test, and then monitored levels of undercarboxylated osteocalcin and reactions to hypoglycemia at regular time intervals. We found differences in the reaction to hypoglycemia between the sexes. In men there was a significant decline in undercarboxylated osteocalcin between the 30 and 40 min (p<0.0015), which reflects a reaction to a glycemic decline between 25-30 min, followed by reversal. Low undercarboxylated osteocalcin in men lasted up to 90 min, after which they returned to levels before the test. In women we did not find any significant changes in undercarboxylated osteocalcin levels. Changes in undercarboxylated osteocalcin induced by hypoglycemia indicate a relationship between bones and glucose metabolism. There was an interesting difference between the sexes. However, a definitive conclusion about the role of osteocalcin in human metabolism will require numerous future studies.

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Introduction

Leptin, the first known hormone from adipose tissue, was discovered more than twenty years ago, and since then evidence has gradually accumulated supporting the hypothesis of adipose tissue as an endocrine organ associated with regulating energy metabolism (Friedman 2014). Recently, there has been an increase of studies reporting on connections between bones and glucose metabolism and reproduction. On the basis of a combination of animal models and clinical observations, bones have thus also been hypothesized to be an endocrine organ (reviewed in Žofková 2015, Zoch *et al.* 2016, Karsenty *et al.* 2016, Stárka and Dušková 2019). The fundamental question remains why bone tissue should be involved in the regulation of energy metabolism. It seems that one reason is that constant bone remodeling is energy-demanding, and its need is even greater during bone growth and repair after damage.

The first hormone to be recognized as being produced by bones is osteocalcin (OC), which had previously been considered just a marker of new bone formation. Osteocalcin (also known as bone Gla-protein) was independently discovered by two teams in 1975 (Hauschka *et al.* 1975, Price *et al.* 1976). Levels of serum osteocalcin correlate with new bone formation and the numbers of osteoblasts (Brown *et al.* 1984). To clarify the role of osteocalcin in bones, OC-deficient (Oc-/-)

animal models were developed, but surprisingly Oc-/- mice did not seem to have any major bone deformities (Ducy *et al.* 1996). In 2007, more detailed analyses of the Oc-/- mouse phenotype produced unexpected results: these mice were found to have hyperglycemia and hypoinsulinemia with reduced insulin secretion compared to wild type mice. In addition, Oc-/- mice had decreases in the size of the islets of Langerhans, numbers of β-cells, pancreatic insulin content, and immunoreactive insulin. Furthermore, Oc-/- mice had higher adipose mass, numbers of adipocytes, and higher insulin resistance in their livers, muscles, and white adipose tissue (Lee *et al.* 2007). That study also focused on genes encoding for secretory or signaling molecules specific for osteoblasts. They found one very interesting gene that is expressed only in osteoblasts and Sertoli cells – Ptpry (Esp) – encoding for osteotesticular protein tyrosine phosphatase (OST-PTP). This finding led to the hypothesis of bones as an endocrine organ that is associated with regulating glucose metabolism and male fertility.

However, currently there are still many unknowns, and opposed to adipose tissues, where endocrine function have been demonstrated in detail and many adipose tissue hormones discovered, the issue of the endocrine function of bones has more questions than answers (Oldknow *et al.* 2015). In addition, there are evident differences between total osteocalcin and undercarboxylated osteocalcin in effects on glucose metabolism. For instance, Ivaska *et al.* (2015) found changes in the ratio of total to undercarboxylated osteocalcin under conditions of hyperinsulinemia during a euglycemic hyperinsulinemic clamp.

The aim of this study was to monitor changes in undercarboxylated osteocalcin in reaction to hypoglycemia, and identify differences in the reaction between the sexes.

Methods

The study included 61 healthy probands with physiological calciophosphate metabolism (characteristics are listed in Table 1). These probands did not take any medications and had no corticoid or hormonal therapy in their medical histories. They had no endocrine or bone disease, and before entering the study signed informed consent. The study was approved by the Ethical Commission of the Institute of Endocrinology. They were given an insulin tolerance test (ITT), which is a functional test that is used to diagnose hypocortisolism.

The test was performed in a fasting state between 7 am and 9 am. A cannula was inserted into the cubital vein to administer the test solution, and the test was started after resting lying down for 15 min.

At the start of the test, 0.1 IU Actrapid per 1 kg body weight was applied intravenously. During the test controls of glycemia (using an Accu-Chek Perform glucometer), blood pressure and pulse were performed every 5 min for the first hour and every 10 min thereafter. In all tests there was a decline in glycemia below 2.2 mmol/l around 30 min, and all probands had a spontaneous reversal of glycemia during the first hour, gradually returning to normal. Blood samples were taken in the time intervals: 0, 20, 30, 40, 60, 90, and 120 min. Blood samples were collected in collection tubes with K2EDTA, immediately centrifuged (5 min, 2000g, 4 °C), and plasma was stored at -80 °C until analysis.

Undercarboxylated osteocalcin measurements

Undercarboxylated osteocalcin was measured using Enzyme Immunoassay Kit (catalogue number MK118) from Takara Bio Inc. This kit specifically measures undercarboxylated osteocalcin with 5.0 % cross-reactivity with human bone osteocalcin (likely Gla type).
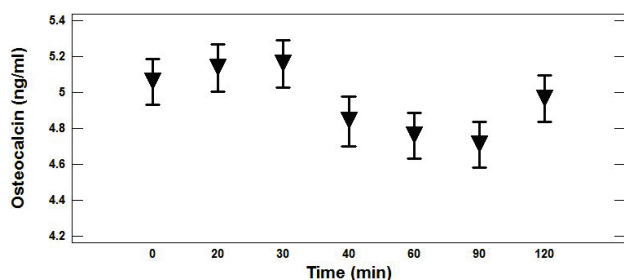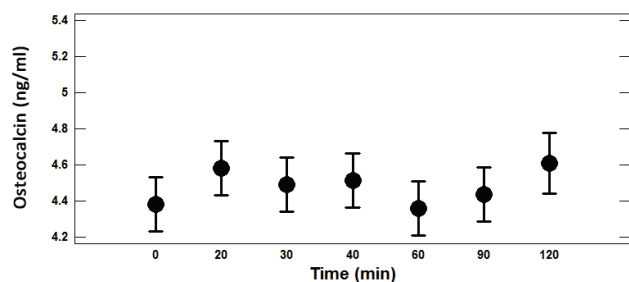
Statistical analysis

Data were transformed by Box-Cox transformation before further processing due to the non-Gaussian distribution and non-constant variance (heteroscedasticity) of all the variables. The repeated measures analysis of variance (ANOVA) model was used for analyzing levels of osteocalcin. The symbols (circles for women and triangles for men) with error bars represent re-transformed means with their 95 % confidence intervals. The 95 % confidence intervals were computed using the least significant difference multiple comparisons (p<0.05). Confidence intervals that did not overlap each other denote significant differences between the respective subgroup means. The statistical software Statgraphics Centurion XVI from Statpoint Inc. (Warrenton, VA, USA) was used for both data transformations and ANOVA.

Results

In our study we found a significant difference of basal levels of undercarboxylated osteocalcin between men and women (Table 1), which lasted throughout the entire test.

Table 1. Characteristics of the study group and basal levels of undercarboxylated osteocalcin.

	Men	Women
n	30	31
Age (mean/median)	37.7/36	44.84/44
BMI (mean/median)	26.55/25.55	24.55/23.63
Basal undercarboxylated osteocalcin (mean ± SD, ng/ml)	5.23 ± 1.96	4.38 ± 1.87

Fig. 1. Changes in the levels of undercarboxylated osteocalcin in men in reaction to hypoglycemia (occurring between 25 and 30 min). There was a significant decline in osteocalcin between 40 and 90 min. Means and 95.0 percent LSD intervals.

Fig. 2. Changes in the levels of undercarboxylated osteocalcin in women in reaction to hypoglycemia (occurring between 25 and 30 min). Means and 95.0 percent LSD intervals.

The reaction to hypoglycemia between sexes significantly differed. In men, we found a significant decline in undercarboxylated osteocalcin between 30 and 40 min ($p < 0.0015$), reflecting a reaction to a glycemia decline between 25 and 30 min followed by reversal. Undercarboxylated osteocalcin in men lasted until 90 min, when levels returned to those before the test (Fig. 1). The glycemia decline was shorter, returning to normal by 60 min. In women we found no significant changes in undercarboxylated osteocalcin levels (Fig. 2).

Discussion

The bone protein osteocalcin (or bone Gla-protein) is a non-collagenous protein secreted by osteoblasts and odontoblasts in the final phase of their differentiation. The protein is an important factor in bone mineralization, and levels reflect the extent of bone mass remodeling. It is composed of 49 amino acids and has a molecular mass of about 5800 Da. Osteocalcin is coded by the bone γ-carboxyglutamate gene, with transcription regulated by the Runx2/Cbfa1 regulatory element. The production of osteocalcin is stimulated by vitamin D and calcitriol, and is dependent on vitamin K. The active form of osteocalcin contains three residues of γ-carboxyglutamic acid, which are critical for its function in bones since they mediate the binding of calcium to osteocalcin. Upon decarboxylation by the enzyme γ-decarboxylase, there is a lowered affinity of osteocalcin to hydroxyapatite. The main function of osteocalcin is to ensure the binding of calcium mineral components in bones, which occurs through two pathways: the regulation of bone mineralization, and regulation of the activity of osteoblasts and osteoclasts.

Some non-bone related changes were already described in osteocalcin-deficient mice by physiologists at the turn of the century, but higher interest began in 2007 with a study on osteocalcin-knock-out mice (Lee *et al.* 2007). That study focused on the regulation of glucose and adipose tissue metabolism mediated by the influence of pancreatic beta cell proliferation and insulin secretion, and in adipose tissue induced by adiponectin production. Effects were induced by just the decarboxylated form of osteocalcin. Since that time, the non-bone related effects of osteocalcin have been the subject of intense research interest (Moser 2019), as have the contrary effects of insulin levels on events in bones and on the regulation of osteocalcin production in osteoblasts (Bilott *et al.* 2018).

The above-mentioned experiments on osteocalcin-deficient mice (Ducy *et al.* 1996, Lee *et al.* 2007) demonstrated that the effects of a lack of osteocalcin on bone mineralization were not as marked as the significant effects on increased glycemia, higher numbers of adipocytes, and increased adipose tissue. Later experiments showed that the decarboxylated form of osteocalcin influences energy metabolism by inducing the production of insulin in beta-cell islets of Langerhans and adiponectins in adipocytes (Lee *et al.* 2007, Oury *et al.* 2013, Wei and Karsenty 2015). Evidence that a lack of insulin receptors in osteoblasts leads to a similar outcome as osteocalcin deficiency supported the idea of a positive feedback loop between insulin and osteocalcin,

with osteocalcin supporting insulin secretion and increased insulin increasing the level of osteocalcin (Fulzele *et al.* 2010).

In contrast to results from animal experiments, our knowledge of the non-bone functions of osteocalcin in humans is comparatively limited. Osteocalcin stimulates the proliferation of human beta cells *ex vivo*, are negatively correlated with the amount of fat in the human body, and are positively correlated with insulin sensitivity and levels of adiponectins (Moser and van der Eerden 2019). Polymorphism in the osteocalcin Bglap locus is associated with type 2 diabetes and obesity. Clinical evidence of the effects of osteocalcin in glucose metabolism can be found in casuistic reports on the results of the ablation of osteoid osteomas that produce osteocalcin. After removing such tumors in two patients, there were significant declines in the levels of osteocalcin by 62 % and 30 %, and glucose levels increased by 32 % and 15 % (Confavreux *et al.* 2012).

The insulin tolerance test is the gold standard used in endocrinology for diagnosing hypocorticalism (Kosák *et al.* 2017). During this test, hypoglycemia leads to the secretion of counter-regulatory hormones that lead to the normalization of glycemia. The increase in cortisol secretion is similar in both sexes (Šimůnková *et al.* 2015), and plays one of the main roles in this glycemic normalization. Since the role of osteocalcin in glucose metabolism is currently a subject of great interest, and since evidence of the function of this mechanism in humans is still lacking, we decided to focus on the reaction of undercarboxylated osteocalcin using this test. We demonstrated a decline in undercarboxylated osteocalcin in men, but not in women. Our data for men are in line with the study of Clowe *et al.* (2002), who found declining bone markers during a hypoglycemic clamp in a group of sixteen men.

We were unable to find any data in the literature on the differences in osteocalcin between the sexes except its effects on fertility, where differences can be attributed to the presence of the GPRC6A receptor, which is a mediator of osteocalcin activity. This receptor is found in Leydig cells, but not in ovarian follicles (Han *et al.* 2018, Oury *et al.* 2011). In men, osteocalcin increases the secretion of testosterone (Oury *et al.* 2013), but other studies did not confirm that it is completely independent of luteotropic hormone (Coskun *et al.* 2019). Testosterone increase is mediated by GPRC6A. Osteocalcin admission cause a significant increase in the

expression of GPRC6A and insulin-like factor 3 by inducing Leydig cell function, but not increase the number of Leydig cells (Coskun *et al.* 2019).

The reason that osteocalcin effects fertility in men remains unclear. However, and the reason that there was no reaction in women is speculative at this point. On possibility is that another form of osteocalcin could play a role (e.g. the level of carboxylation) in an acute reaction in women to changes in glycemia, or another hormone entirely could be involved. We could hypothesize of osteotesticular protein tyrosine phosphatase role, which is may regulated carboxylation of osteocalcin (Kim *et al.* 2010).

Osteocalcin also has distinct effects on cognitive function, as has been shown in both mice and humans (Oury *et al.* 2013). Such effects of osteocalcin on brain function could be the connection that centrally provides information on bone status and influences it´s remodeling. Bone remodeling is energy-demanding, and thus influences glucose metabolism. Hypoglycemia is a life-threatening situation, and the primary target of damage is the brain. The brain therefore immediately reacts with mechanisms to increase glycemia, one of which is the glucocorticoid activity of cortisol, but also suppresses the secretion of osteocalcin, which in humans helps increase glycemia (Confavreux *et al.* 2012). However, such a definitive conclusion on the function of osteocalcin in human metabolism will require many future studies.

In conclusion, we found differences between the sexes in the reaction of undercarboxylated osteocalcin to hypoglycemia. In men we demonstrated a decline that lasted 50 min, after which osteocalcin returned to basal levels. In women we saw no significant changes in the response of undercarboxylated osteocalcin to hypoglycemia. Sex differences in undercarboxylated osteocalcin response to insulin tolerance test are further indirect evidence for the involvement of osteocalcin in male reproduction.

Conflict of Interest
There is no conflict of interest.

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