OSTEOCALCIN IN A RAT MODEL OF METABOLIC SYNDROME

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Abstract

Osteocalcin (OC) is an osteoblast-derived vitamin K dependent protein. Recent studies have demonstrated that in mice it acts as a multifunctional hormone, increasing insulin secretion and beta cell proliferation and improving insulin sensitivity. Osteocalcin has been postulated to be metabolically active in its undercarboxylated form. Whether OC plays a similar metabolic role in the rat is currently unknown. The aim of the present study was to compare the levels of OC in intact rats and in rats with a diet-induced metabolic syndrome. A secondary objective was to look for correlations of OC with metabolic parameters. Two groups of rats were used. The control group received regular rat chow and plain water and the experimental group was fed a diet high in saturated fat and fructose. The duration of the experiment was 10 weeks. At the end of this period insulin tolerance test was performed. Serum lipids, insulin, leptin and both forms of OC, carboxylated and undercarboxylated, were determined. Metabolic syndrome was verified by increased visceral adiposity, elevated serum triglycerides, cholesterol and fasting blood glucose, positive insulin tolerance test at the 90th minute, higher serum insulin. In rats with metabolic syndrome undercarboxylated OC serum level was significantly reduced, whereas carboxylated OC level was unchanged. The ratio undercarboxylated OC/ carboxylated OC was also lower in the experimental group. Undercarboxylated OC and undercarboxylated OC/ carboxylated OC were inversely associated with blood glucose but not with other biochemical parameters. Our results partly support the hypothesis that undercarboxylated OC is implicated in energy regulation and suggest that this might be true also for the rat. More evidence is needed to determine the hormonal role of osteocalcin in this animal species.

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Introduction

Osteocalcin (OC) is a protein typical for bones. It was isolated in 1975 by two independent research groups (1, 2). It is produced by osteoblasts, incorporated in bone matrix and released in circulation through the process of bone resorption. Traditionally OC is regarded as a marker of bone formation (3). It is also known as bone Gla-protein since its molecule contains three gamma-glutamic acid residues that undergo vitamin K dependent gamma-glutamic carboxylation. In this way, the undercarboxylated OC is converted to its carboxylated form. In general, OC is considered, like the other vitamin K dependent proteins (e.g. those involved in coagulation), as active in its carboxylated form, vitamin K being essential for its functional activity in the bone.

The classical understanding about the role of OC in skeleton was shaken when a research group led by Gerard Karsenty at Columbia University, New York, NY, USA demonstrated in experiments on OC knock-out mice that they had virtually normal bones. Unexpectedly, however, these mice consistently developed obesity phenotype with glucose intolerance and insulin resistance. It turned out from these and later studies that, at least in mice, OC exerted metabolic functions (4). Thus, OC has been qualified as a hormone (osteokine) secreted by bone and involved in the regulation of energy homeostasis through actions on pancreatic beta cells and adipocytes. According to this hypothesis, OC is decarboxylated during bone resorption and released into the systemic circulation in its undercarboxylated form. Therefore, it is the undercarboxylated OC that exerts a hormonal action. The release of undercarboxylated OC into the blood is under the positive control of insulin (5) and is negatively regulated by leptin (6). Metabolic effects of undercarboxylated OC include increased insulin secretion and pancreatic beta cell proliferation and enhanced insulin sensitivity in adipose tissue and skeletal muscles (4, 7). Treatment of mice subjected to high-fat diet with undercarboxylated OC improves insulin sensitivity and glucose tolerance, reduces plasma triglycerides and protects the animals from diet-induced obesity and hepatic steatosis (7, 8).

The intriguing new findings suggesting a role for OC in obesity, metabolic syndrome and type 2 diabetes mellitus was reflected by a number of clinical and epidemiological studies undertaken to test this hypothesis in humans. Early clinical observations considered total OC and found associations with parameters of glucose and lipid metabolism in individuals with type 2 diabetes (9-11) or metabolic syndrome (12-15). The relationships between undercarboxylated OC and energy homeostasis have been less investigated so far and the results are controversial. Some studies support the hypothesis that undercarboxylated OC is the active metabolic form of OC (16-20). Others demonstrate metabolic activity of carboxylated OC as

well (19, 21). Still others find no relationship between undercarboxylated OC and carbohydrate homeostasis (21, 22). The heterogeneity of results in humans poses the question about possible variability of OC actions in different animal species.

Whether OC serves a metabolic role in the rat is currently unknown. The aim of the present study was to compare the levels of OC in intact rats and in rats with diet-induced metabolic syndrome and to look for correlations of both forms of OC with biochemical measures of glucose and lipid homeostasis.

Materials and Methods

Experimental animals

The study was performed on 20 male Wistar rats. The animals were kept at an ambient temperature of 20-25 °C and 12-hour light-dark cycle. They had free access to food and water. The study was approved by the Commission of foods safety in the Ministry of Agriculture and Foods and was conducted in agreement with the National Policies and the Council Directive (86/609/EEC).

The rats were allocated into two groups of 10 rats each, with initial average body weight of 258 g in both groups. The duration of the study was 10 weeks. The first group was a control group (C) and the second group was an experimental group with diet-induced metabolic syndrome.

Diets

The rats from the C group were fed a standard rat chow and were given plain water to drink. With each 100 g food consumed, these animals received 279 kcal. The second group of rats was subjected to a diet-induced model of metabolic syndrome (23). They were given a diet with lard (17%) and fructose (17%) added to the standard rat chow and a 10% fructose solution to drink instead of water. The caloric intake was 405 kcal per 100 g food of which 38% was provided by lard and 17% by fructose. These animals received also additional 40 kcal per 100 ml of the fructose solution consumed. Body weight of the animals was measured every other day. At the end of the experiment insulin tolerance test and biochemical tests were performed.

Insulin tolerance test

Insulin tolerance test (ITT) was carried out after 4 hours of fasting. Regular insulin (ActRapid), dissolved in saline, was used at a dose 0.75 UI/kg. Animals were injected i.p. with 0.2 ml/100 g body weight insulin solution. Blood glucose was measured by a glucometer (ACCU-CHEK Performa). Blood samples were taken by incision of the distal part of the tail (24) immediately before the injection of insulin (at time 0) and at the 30th, 60th and 90th minute. The blood glucose levels at time 0 will be con-

sidered as fasting blood glucose in the further text. Blood for biochemical tests was taken from the sublingual veins of the animals under ether anesthesia. The blood was centrifuged and the serum was refrigerated at -20°C until biochemically tested. The rats were euthanized by cervical dislocation. The abdomen was opened and the right retroperitoneal fat pad was dissected out, weighed and the fat index was calculated as a ratio of fat weight to body weight $\times 10^3$.

Biochemical tests

Serum triglycerides and cholesterol were measured by using colorimetric kits of HUMAN, LINEAR CHEMICALS S.L., Barcelona, SPAIN, at a spectrophotometer AURIUS 2021 (Cecil Instruments Ltd.). Serum insulin and leptin, as well as serum levels of both forms of OC – undercarboxylated and carboxylated, were measured by ELISA assay kits (Rat Insulin ELISA Kit of Shibayagi, Rat Leptin ELISA Kit of BioVendor, Rat Glu-Osteocalcin and Rat Gla-Osteocalcin ELISA Kits of Takara), following the producer's instructions. The ratio undercarboxylated OC/ carboxylated OC was calculated.

Statistics

Results are presented as a mean $\pm$ standard error of the mean (SEM). The two groups were compared by Student's unpaired t-test. Associations between OC levels and metabolic parameters were analyzed by correlation analysis. Differences were considered significant at $p < 0.05$. The statistical software GraphPad Prism 5 was used (GraphPad Software, Inc.).

Results

After 10 weeks of diet manipulation the body weight of the animals from the experimental group did not differ significantly from that of controls. The metabolic syndrome induced by the diet was verified by increased visceral adipose tissue (fat index),

elevated serum triglycerides, cholesterol and fasting blood glucose, positive ITT at the 90th minute, higher insulin and leptin levels (Table 1). Changes in OC levels are shown on Figure 1. In the rats with metabolic syndrome, the level of undercarboxylated OC (Fig. 1A) was decreased: 27.12 ± 2.287 ng/ml in the MS group compared to 40.31 ± 4.674 ng/ml in the C group ($t=2.535$, $df=16$, $p=0.0221$). The carboxylated OC level (Fig. 1B) was insignificantly increased: 577.2 ± 49.96 ng/ml in the metabolic syndrome group compared to 486.1 ± 39 ng/ml in the C group ($t=1.436$, $df=16$, $p=0.1702$). The ratio undercarboxylated OC/ carboxylated OC (Fig. 1C) was also reduced in the experimental group: 0.03957 ± 0.0037 in the MS group compared to 0.08163 ± 0.0034 in the C group ($t=8.341$, $df=13$, $p<0.0001$).

Table 1. Verification of metabolic syndrome – metabolic characteristics of animals from the control and the metabolic syndrome groups; * $p < 0.05$ vs C

Parameter \ Group	Control	Metabolic syndrome
Final body weight (grams)	374.4 ± 8.2	353.0 ± 8.6
Fat index	7.94 ± 0.77	$11.71 \pm 1.25^*$
Triglycerides (mmol/l)	1.626 ± 0.07	$1.967 \pm 0.13^*$
Cholesterol (mmol/l)	2.469 ± 0.04	$2.631 \pm 0.06^*$
Fasting blood glucose (mmol/l)	5.97 ± 0.11	$6.48 \pm 0.14^*$
ITT at the 90 th minute (% of initial value)	38.1 ± 1.75	$44.3 \pm 1.87^*$
Fasting insulin (ng/ml)	0.79 ± 0.11	$1.53 \pm 0.24^*$
Leptin (pg/ml)	16.5 ± 4.86	49.8 ± 18.16

Figure 1. Changes in osteocalcin (OC) levels in rats with metabolic syndrome (MS) compared to control rats (C); **A.** Changes in serum level of undercarboxylated osteocalcin (ucOC). **B.** Changes in serum level of carboxylated osteocalcin (cOC). **C.** Changes in the ratio between undercarboxylated and carboxylated OC (ucOC/cOC). * $p < 0.05$, *** $p < 0.0001$ vs C.

Correlation analysis (Fig.2) revealed that the level of undercarboxylated OC was negatively associated with fasting blood glucose (Fig. 2A): Pearson $r = -0.5409$, $p = 0.0250$. The same was true for the ratio undercarboxylated OC/ carboxylated OC (Fig. 2B): Pearson $r = -0.6644$, $p = 0.0096$.

No associations were found between undercarboxylated OC and visceral adipose tissue, serum insulin, leptin, triglycerides and cholesterol levels. Carboxylated OC showed no correlations with any of the metabolic parameters examined.

Discussion

The results from the present study demonstrate that OC is most probably involved in the regulation of energy homeostasis in rats, similarly to its actions in mice. Obviously, it is the undercarboxylated form of the protein that is likely endowed with hormonal activity.

The diet high in saturated fat and fructose induced in the experimental rats a state similar to metabolic syndrome in humans, evidenced by somatic and biochemical measures. Insulin resistance was presented by increased fasting level of serum insulin and impaired response to insulin at the 90th minute of the ITT. The reduced insulin sensitivity affects not only the adipose tissue, liver and skeletal muscles, but also the insulin receptors on osteoblasts. In mice increased uptake of saturated fatty acids by osteoblasts accelerates the ubiquitination and degradation of insulin receptors (25). Insulin signaling in osteoblasts is responsible for the regulation of OC secretion in the circulation (5). Activation of insulin receptors suppresses the production of osteoprotegerin, an inhibitor of osteoclast maturation. The bone resorption is thus stimulated, providing the acidic environment needed for the decarboxylation and the activation of OC. Insulin resistance, as seen in MS, impairs this regulatory

mechanism and results in reduced circulating level of undercarboxylated OC. This is what we observed also in our experiment in rats: the concentration of undercarboxylated OC was reduced in the rats fed energy-dense diet compared to controls. Unlike undercarboxylated OC, carboxylated OC level was not affected by the diet manipulation. It was only slightly and insignificantly elevated. These results suggest that carboxylated OC is not involved in the regulation of energy homeostasis, whereas undercarboxylated OC exerts hormonal activity in rats. It appears that the ratio between the undercarboxylated and carboxylated OC even better reflects the diet-induced metabolic alterations in the rats in our experimental setting.

Increased leptin levels may also contribute to the decrease of serum undercarboxylated OC level since leptin is another endocrine factor involved in the regulation of its release (6). By stimulating the sympathetic tone, leptin leads to activation of β 2-adrenergic receptors on osteoblasts, finally resulting in reduced release of undercarboxylated OC. In our experiment, however, leptin was probably not implicated in the decrease of undercarboxylated OC, since its increase was not statistically significant. On the other hand, higher leptin level can be regarded as a feature of the metabolic syndrome itself with development of resistance, where the effect of leptin on undercarboxylated OC might be blunted.

All the cited experimental studies concerning the hormonal role of undercarboxylated OC have been carried out on mice – genetically modified in different ways and wild type mice.

Our results are the first to show that in the rat OC may have a metabolic role too. In addition to the decreased level of undercarboxylated OC and the lower ratio between undercarboxylated and carboxylated OC in the MS rats, this notion is further reinforced by the inverse correlations that we found between

Figure 2. Correlations of: **A.** Undercarboxylated OC (ucOC) with fasting blood glucose and **B.** Undercarboxylated to carboxylated OC ratio (ucOC/cOC) with fasting blood glucose.

these variables and the fasting blood glucose. Ferron *et al* (8) have shown that in mice undercarboxylated OC is involved in the pathogenesis of metabolic disorders. They demonstrated that mice fed high-fat diet can be protected from type 2 diabetes by daily injections of undercarboxylated OC. Under the conditions of diet manipulation, undercarboxylated OC is probably involved in a vicious cycle – insulin resistance leads to decreased release of undercarboxylated OC and the reduced undercarboxylated OC level results in further impairment of insulin sensitivity. The negative association between undercarboxylated OC and the fasting blood glucose that we have observed seems a logical consequence of the metabolic derangement following diet loading. Furthermore, carboxylated OC correlated neither with blood glucose nor with any other metabolic or somatic parameter. However, we did not find any correlation of undercarboxylated OC with biochemical measures other than blood glucose. We could speculate that undercarboxylated OC in rats is less metabolically active than in mice.

While our results partly replicated those in mice, they appear to be closer to those results observed in humans that are in line with the murine. In general, the data from the clinical observations are very inconsistent. In earlier studies the *total* OC has been investigated and negative associations with blood glucose have been reported in patients with type 2 diabetes or metabolic syndrome (9-15). In these studies OC was also positively or negatively correlated with a variety of other variables, such as insulin and/or adiponectin levels, insulin resistance and/or sensitivity measures, serum triglycerides and HDL-cholesterol, body mass index, waist circumference and blood pressure.

The undercarboxylated form of OC in healthy individuals or in type 2 diabetic patients has been found to be negatively associated with blood glucose (16, 19, 20), similarly to what we have observed in rats. In other studies undercarboxylated OC was positively correlated with insulin sensitivity (17), adiponectin levels (18) and pancreatic beta cell function (19).

Some epidemiological studies suggest that in humans *both* forms of OC are involved in regulation of energy homeostasis. According to Hwang *et al* undercarboxylated OC was associated with enhanced beta cell function, while carboxylated OC correlated with improved insulin sensitivity (19). Other authors found that low levels of carboxylated, but not undercarboxylated OC, were associated with insulin resistance (21). Further on, it has been reported that the decrease of undercarboxylated OC in response to vitamin K supplementation had no effect on insulin resistance suggesting that this form of OC is not involved in the regulation of glucose metabolism in humans (22).

Our results in rats are discordant with the reported endocrine activity for carboxylated OC in humans and with the lack of effect

of its undercarboxylated form, rather, they support the hormonal function of the undercarboxylated OC on the energy homeostasis. We may thus propose that, despite the firm evidence about the hormonal role of undercarboxylated OC in mice, in humans the results are not so homogeneous in their agreement as to whether and which form of OC is hormonally active in regulating energy homeostasis. Combined with our modest results on rats, the data thus far seem to point to the possibility that OC might not behave in the same manner in different animal species.

Conclusion

The present study provides some support to the hypothesis that OC is implicated in energy regulation in the rat. Similarly to mice, the undercarboxylated OC was the form that was implicated in the metabolic changes in the diet-manipulated rats. However, compared to its effects in mice, the metabolic activity of undercarboxylated OC in rats might turn out to be more limited. Given the contradictory results that have been reported from human studies looking at the role of OC in regulation of energy homeostasis, it can be inferred that species differences probably exist in its hormonal activity. Definitely much more work is needed to convincingly determine whether OC is involved in metabolic regulation in the animal kingdom as a whole.

Conflicts of interest

The authors declare no conflicts of interest.

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