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Correlations of Carboxylated and Un(der)carboxylated Osteocalcin with Parameters of Energy Metabolism in Subjects with and without Type 2 Diabetes

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Abstract

Background: Osteocalcin is a vitamin K-dependent protein produced by osteoblasts. In the systemic circulation it is found in both carboxylated and un- or undercarboxylated form. Experimental studies have revealed that un(der)carboxylated osteocalcin affects beneficially the energy metabolism in mice and rats. Clinical trials have confirmed the endocrine activity of osteocalcin in humans. However, it remains controversial as to which form of the protein is the one possessing hormonal function.

Aim: In an attempt to clarify the above question, we searched for associations of un(der)carboxylated and carboxylated osteocalcin with anthropometric and biochemical parameters in subjects with and without type 2 diabetes.

Methods: The current cross-sectional study included 46 diabetics and 19 non-diabetic participants. Circulating levels of carboxylated and un(der)carboxylated osteocalcin were measured through high-sensitive enzyme immunoassay kits. The energy metabolism was characterized by anthropometric measures, lipid profile, fasting blood glucose and glycated hemoglobin level. The level of lipid peroxidation was determined to assess oxidative stress.

Results: Carboxylated osteocalcin was negatively correlated with body mass index, waist circumference and waist-to-height ratio and positively associated with HDL-cholesterol in the entire sample and in the non-diabetic group. In the diabetic group carboxylated osteocalcin was associated with waist circumference and HDL-cholesterol. Un(der)carboxylated osteocalcin was negatively correlated with fasting blood glucose in the non-diabetic group.

Conclusion: Our study confirmed the connection of osteocalcin with parameters of metabolic disorders in humans. We found associations indicating beneficial metabolic effects for both forms of osteocalcin, the carboxylated one being more active.

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Introduction

Osteocalcin (OC) is a bone-derived protein, containing three gamma-glutamic acid residues. After its synthesis in osteoblasts, it undergoes posttranslational vitamin K-dependent carboxylation of all three gamma-glutamic acid residues [1]. The traditional understanding of OC role postulates that the carboxylated form of the protein (cOC) is essential for normal bone structure and function. Circulating levels of OC are measured to predict the fracture risk and to assess the effectiveness of antiresorptive therapy [2]. In the process of bone resorption, one or more of the carboxylated residues are decarboxylated due to the acidification of the environment, and the protein is released in the systemic circulation as un- or undercarboxylated OC (ucOC) [3].

A number of experimental studies conducted on wild type and OC-deficient mice over the past 20 years have shown that ucOC plays an important role in the regulation of energy metabolism by stimulating insulin release and increasing peripheral insulin sensitivity [4,5]. In our previous work, we have shown that ucOC is hormonally active also in rats [6]. We found that ucOC level was reduced in rats with diet-induced metabolic syndrome, while cOC remained unchanged. In addition, there was a negative correlation between ucOC concentration and fasting blood glucose level [6]. We next showed that the pharmacological reduction of ucOC, achieved by alendronate administration, was associated with impairment of glucose metabolism [7]. In this study, ucOC level was negatively correlated with the fat index of the experimental animals as an indicator of visceral obesity. Our findings in rats confirmed that, similarly to mice, the un(der) carboxylated form of OC appeared to possess hormonal activity, while the carboxylated form was inactive.

The newly discovered hormonal role of OC in rodents has attracted scientific interest and has stimulated numerous clinical trials to test this activity in humans. In general, the results are in agreement

with the experimental evidence. Several reviews and meta-analyses have concluded that the level of OC is lower in patients with type 2 diabetes and total OC (tOC) correlates with numerous parameters of energy metabolism [8,9]. However, trials studying the significance of the carboxylation state of the protein have reported contradictory results [10-12]. It remains thus unclear which of the forms of OC possesses endocrine activity in humans – the un(der)carboxylated, the carboxylated, or both.

In the current study we aimed to clarify which is the metabolically active form of OC in humans. Therefore, we searched for associations of ucOC and cOC with various anthropometric and biochemical parameters in patients with type 2 diabetes and non-diabetic subjects.

Methods

Participants

The current cross-sectional observational study included a sample of 46 patients with type 2 diabetes (25 females and 21 males) of age 58.1 ± 7.6 (mean ± SD), and a control group of 19 subjects without diabetes (11 females and 8 males) of age 57.4 ± 6 (mean ± SD). Anthropometric measures of general and central obesity, such as body mass index (BMI), waist circumference (WC) and waist-to-height ratio (WHtR), were taken. Blood samples were obtained for biochemical analyses. All participants gave written informed consent prior to the enrolment. The study was approved by the local Ethical Committee of the Medical University of Varna.

Measurement of Osteocalcin Concentration

Serums of the participants were collected and stored at -60°C for biochemical measurement of OC concentration. High sensitive sandwich-type enzyme immunoassay (EIA) kits for ucOC and cOC were used (Takara Bio, Inc., Japan), following the producer's instructions. Both kits utilize two mouse monoclonal antibodies against OC, one of which is coated onto the plate and the other is peroxidase-labeled. The first recognizes specifically the measured protein (ucOC or cOC respectively). The peroxidase-labeled anti-OC monoclonal antibody is required for the reaction between peroxidase and the substrate added resulting in color development with intensities proportional to the respective amounts of ucOC and cOC present in the samples. The amount of the proteins was quantitated by measuring the absorbance using an ELISA reader LKB 5060-006 (LKB Instruments, Australia) at 450 nm. Total OC level was calculated as a sum of ucOC and cOC concentrations.

Biochemical Parameters

Standard biochemical parameters of energy homeostasis were measured in the blood samples of the participants. The carbohydrate metabolism was evaluated by fasting blood glucose (FBG) and glycated hemoglobin (HbA1c) level. Lipid profile (triglycerides, LDL-cholesterol, HDL-cholesterol, and total cholesterol) was determined. All analyses were performed immediately after blood sampling by routine laboratory methods in the clinical laboratory of St. Marina University Hospital, Varna, Bulgaria.

Serum thiobarbituric acid reactive substances (TBARS) reflecting lipid peroxidation were measured to evaluate the level of oxidative stress. TBARS were determined colorimetrically at 532 nm by using the method of Ohkawa H et al., (1979) [13] with Aurius 2021 UV-VIS spectrophotometer (Cecil Instruments Ltd, UK).

Statistics

The groups were compared by Student's two-tailed unpaired t-test. The associations of cOC and ucOC levels with biochemical and anthropometric parameters were tested by correlation analysis. A level of $p<0.05$ was considered significant. GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) statistical software was used.

Results

The anthropometric and biochemical parameters of the participants, as well as OC concentrations are presented in the below table [Table 1]. FBG and HBA1c levels were higher in the diabetic vs. the non-diabetic group. The circulating concentrations of cOC and ucOC, as well as calculated tOC, were lower in the group of diabetic patients compared to the non-diabetic subjects.

The associations of both forms of OC with anthropometric and biochemical parameters of energy metabolism are presented in the below table [Table 2].

When we analyzed all the participants in the study, we found that the carboxylated form of the OC was negatively correlated with the anthropometric parameters of general and central obesity and positively associated with HDL-cholesterol level. The correlation analysis revealed a borderline significance for the negative association between cOC and FBG. All correlations of cOC remained significant when we analyzed separately the non-diabetic group (Figure 1A). Among the diabetic patients, cOC was only correlated with WC and HDL-cholesterol (Figure 1B). The association with BMI was of borderline significance.

Table 1: Anthropometric and biochemical parameters (mean ± SD).

		Non-diabetic subjects	Diabetic subjects
Anthropometric parameters	BMI	29.7 ± 6.35	32.9 ± 6.8
	WC [cm]	101.8 ± 17.5	108.9 ± 15.1
	WHtR	0.61 ± 0.09	0.65 ± 0.09
Carbohydrate metabolism	FBG [mmol/l]	5.79 ± 0.83	7.71 ± 2.31**
	HBA1c [%]	6.2 ± 0.58	9.34 ± 2.27***
Lipid profile	TG [mmol/l]	2.06 ± 1.34	2.14 ± 1.57
	TC [mmol/l]	5.64 ± 1.55	5.33 ± 1.47
	HDL-C [mmol/l]	1.33 ± 0.36	1.21± 0.41
	LDL-C [mmol/l]	3.26 ± 1.07	3.11 ± 1.19
Oxidative stress	TBARS [nmol/ml]	27.7 ± 8.21	25.6 ± 8.2
Osteocalcin	cOC [ng/ml]	9.84 ± 4.34	7.88 ± 2.42*
	ucOC [ng/ml]	4.3 ± 2.93	2.99 ± 1.8*
	tOC [ng/ml]	13.81 ± 6.01	11.1 ± 3.38*

Whereas: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs non-diabetic subjects; BMI: Body mass index; WC: Waist circumference; WHtR: Wist-to-height ratio; FBG: Fasting blood glucose; HBA1c: Glycated hemoglobin; TG: Triglycerides; TC: Total cholesterol; HDL-C: High-density lipoprotein cholesterol; LDL- C: Low-density lipoprotein cholesterol; TBARS: Thiobarbituric acid reactive substances; cOC: carboxylated osteocalcin; ucOC: un(der)carboxylated osteocalcin; tOC : Total osteocalcin.

Table 2: Correlations of carboxylated (cOC) and un(der)carboxylated osteocalcin (ucOC) with anthropometric and biochemical parameters.

	cOC			ucOC		
	All participants	**Non-diabetic subjects**	**Diabetic patients**	**All participants**	**Non-diabetic subjects**	**Diabetic patients**
BMI	r = -0.333	r = -0.466	r = -0.284	r = -0.143	r = -0.338	r = 0.042
	P = 0.007	**P =0.044**	P =0.0589	P > 0.05	P > 0.05	P > 0.05
WC	r = -0.553	r = -0.560	r = -0.349	r = -0.135	r = -0.335	r = 0.046
	P < 0.001	**P =0.013**	**P =0.019**	P > 0.05	P > 0.05	P > 0.05
WHtR	r = -0.4569	r = -0.488	r = -0.264	r = -0.112	r = -0.316	r = 0.068
	P =0.001	**P =0.034**	P > 0.05	P > 0.05	P > 0.05	P > 0.05
FBG	r = -0.250	r = -0.027	r = -0.193	r = -0.144	r = -0.483	r = 0.057
	P = 0.050	P > 0.05	P > 0.05	P > 0.05	**P = 0.049**	P > 0.05
HBA1c	r = -0.063	r = -0.129	r = -0.044	r = -0.183	r = 0.009	r = -0.141
	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
TG	r = -0.169	r = -0.286	r = -0.215	r = -0.147	r = -0.395	r = -0.053
	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
TC	r = 0.078	r = 0.016	r = 0.154	r = -0.056	r = -0.243	r = -0.126
	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
HDL-C	r = 0.301	r = 0.537	r = 0.429	r = 0.232	r = 0.108	r = 0.022
	P =0.019	**P =0.032**	**P =0.003**	P > 0.05	P > 0.05	P > 0.05
LDL-C	r = 0.118	r = 0.145	r = 0.222	r = -0.098	r = -0.108	r = -0.143
	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
TBARS	r = -0.008	r = 0.077	r = -0.006	r = -0.005	r = 0.037	r = -0.023
	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05

Whereas cOC: Carboxylated osteocalcin; ucOC : un(der)carboxylated osteocalcin; BMI: Body mass index; WC: Waist circumference; WHtR : Waist-to-height ratio; FBG: Fasting blood glucose; HBA1c: Glycated hemoglobin; TG – Triglycerides; TC : Total cholesterol; HDL-C : High-density lipoprotein cholesterol; LDL-C : Low-density lipoprotein cholesterol; TBARS : Thiobarbituric acid reactive substances.

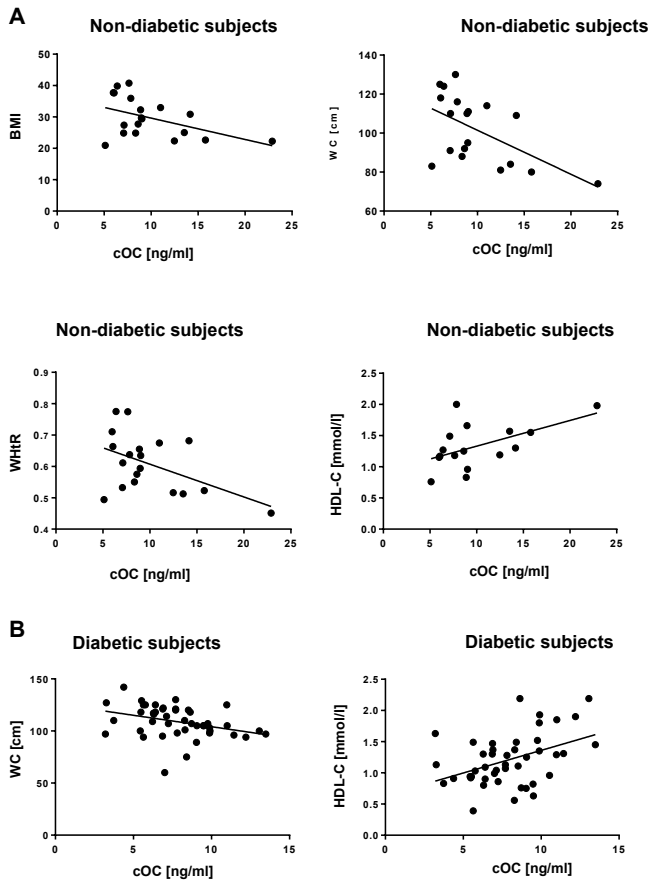

Figure 1: (A) Correlations of carboxylated osteocalcin with parameters of energy metabolism in non-diabetic and (B) diabetic subjects.

Whereas cOC: carboxylated osteocalcin; BMI: Body mass index; WC: Waist circumference; WHtR: Waist-to-height ratio; HDL-C: High-density lipoprotein cholesterol.

The remaining parameters, including TBARS levels, were not related to the cOC concentration. The un(der)carboxylated form of OC did not correlate with most of the anthropometric and biochemical parameters. We found only a negative association with FBG in the control group.

Discussion

In the current study both forms of OC were lower in diabetic patients compared to non-diabetic participants. Thus, the results demonstrate the possible connection of OC to the impairment of glucose metabolism in the investigated subjects. Lower levels of cOC and/or ucOC in patients with type 2 diabetes compared to controls have been reported by other authors [14-16], and a meta-analysis has concluded that tOC is reduced in diabetic patients [8]. The correlation analysis that we performed aimed to reveal if and to what extent the different levels of cOC and ucOC were associated with the metabolic parameters characterizing the two groups.

In our study we found several correlations of cOC with parameters of energy metabolism, all of them pointing to beneficial metabolic effects of carboxylated form of the protein. In contrast, ucOC level did not correlate with most of the measured parameters, the exception being its association with FBG in the control group. Our results are in line with those reported by Knapen et al. In their study they supplemented young men and women, as well as postmenopausal women with vitamin K2, which is responsible for the carboxylation of vitamin K-dependent proteins outside the liver. The authors found that the carboxylation of OC was significantly correlated with lower body weight, BMI and fat mass of the trunk. At the same time, the uncarboxylated form of the protein was not associated with fat mass and body weight [10]. In a study that examined the cross-sectional and longitudinal associations between different forms of OC and insulin resistance in older men and women Shea MK et al., (2009) have received similar results – elevated cOC was associated with lower insulin resistance and ucOC was not [17]. Correlations of cOC, but not ucOC, with insulin resistance and

fasting blood glucose have been reported also by Díaz-López A et al., (2013) in a prospective nested case-control study [14]. Associations of cOC with different anthropometric and biochemical measures, suggesting beneficial metabolic effects of the protein, have been reported in other clinical studies as well [11,18,19]

Some of the above mentioned studies have reported beneficial associations not only of cOC, but also of ucOC [11,18,19]. In addition, there are many authors that have measured the concentration of ucOC without comparing its effects to those of cOC and confirmed the presence of associations for the un(der)carboxylated form [12,20,21]. In most of these investigations ucOC was negatively correlated with parameters of carbohydrate metabolism, such as FBG, glycated hemoglobin level or insulin resistance. The relation between ucOC and carbohydrate metabolism has been confirmed in a meta-analysis by Liu DM et al., (2015) who found that ucOC and tOC correlate strongly and similarly with FBG and glycated hemoglobin level [22]. In our study, we also found a negative association of ucOC with FBG but only in the control group.

The existent discrepancy between the results from different clinical trials is difficult to explain. According to some authors the contradictory data are due to the methodological flaws related to the measurement of the circulating ucOC [23-25]. The molecule of the uncarboxylated OC contains three glutamic acid residues that are located at different positions in rodents and humans. In the human molecule, these positions are the 17th, 21st and 24th [26]. The experimental studies on mice have revealed that the endocrine activity of OC is dependent on the carboxylation status of the first glutamic acid residue in the molecule [3]. Therefore, it is suggested, by analogy, that in humans, it is the carboxylation status of the glutamic acid residue at position 17 that is responsible for the hormonal activity of OC [24]. The molecules that are uncarboxylated only at position 17 are considered as undercarboxylated. According to this concept, the antibodies in the commercially available kits for ucOC cannot properly detect the 17-undercarboxylated OC, thus leading to inaccurate results [23,24]. Recently a novel immunoassay method claiming to precisely detect the circulating level of the active ucOC has been developed [25]. The above understanding, however, is not compatible with the positive findings reported with the use of commercial kits.

Our study confirmed the connection of osteocalcin with parameters of metabolic disorders in humans. We found associations indicating beneficial metabolic effects for both forms of osteocalcin, the carboxylated one being more active.

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Authors'Contributions

All authors contributed to the conception and design of the study; Silvia Gancheva and Maria Zhelyazkova-Savova analyzed and interpreted the results; Silvia Gancheva drafted the manuscript. Maria Zhelyazkova-Savova and Branimir Kanazirev revised it critically; all authors approved the final version of the article; all authors agree to be accountable for all aspects of the work.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

The study is ethically approved by the local Ethical Committee of the Medical University of Varna (No. 79/29.11.2018).

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