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Unacylated ghrelin in plasma of Wistar rats after consumption of fructose, sucrose and aspartame

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Unacylated ghrelin (UAG) amounts to 80-90% of the circulating orexigenic hormone ghrelin. Studies suggest that both acyl ghrelin (AG) and UAG may mediate peripheral biological actions and UAG can act as a potent functional inhibitor of ghrelin. The aim of this study was to track changes in unacylated ghrelin in plasma in a control and three experimental (fructose, sucrose and aspartame) groups of rats and to compare them with some morphological and metabolic parameters. An 8-week burdening of 28 male Wistar rats with 15% fructose (Group F, n = 7), 10% sucrose (Group S, n = 7) and 0.3% aspartame solutions (Group A, n = 7) was carried out. An increase in average body weight was found in the following order: sucrose group > fructose group > aspartame group > controls. Significant difference was found in the mesenteric fat depot weight of Group F vs. Group C. An increase in unacylated ghrelin levels and in general metabolic parameters (glucose, triglycerides, total and LDL-cholesterol, AST, ALT) of the fructose and sucrose groups compared to controls was registered. Moreover, changes in some metabolic markers in the aspartame group were also seen. In conclusion, the results of the current study suggest that elevated unacylated ghrelin might trigger changes in the regulation of food intake and further development of obesity, metabolic disorders and chronic noninfectious diseases.

Keywords: unacylated ghrelin, metabolic syndrome, fructose, sucrose, aspartame, Wistar rats

INTRODUCTION

Excessive consumption of sucrose and fructose (in the form of high fructose corn syrup) is one of the most serious causes of obesity and comorbidities, such as hypertension, diabetes, nonalcoholic fatty liver disease and coronary heart diseases, which are associated with a significant increase in morbidity and mortality rates [1,2,3,4]. In order to reduce sucrose and fructose consumption they are replaced with artificial sweeteners like aspartame. Sweeteners are widely used in many products, such as desserts and diet beverages, as a mean to prevent body mass gain, metabolic syndrome, diabetes, and several risk factors for heart disease. In 2012, the American Heart Association and American Diabetes Association concluded that there are still insufficient data to determine the role played by artificial sweeteners in the regulation of energy balance, body weight, and influence on cardiometabolic risk factors [5]. There is evidence that non-caloric sweeteners increase appetite, promote overeating, and lead to body mass gain [6, 7, 8]. The mechanisms by which this occurs remain unknown. It is probable that a significant role in this mechanism is played by the sweet taste receptors T1R2 and T1R3 (G-coupled receptor proteins), which have also been found in the duodenum, small intestine and pancreas. Stimulation of this taste receptors by sugars or artificial sweeteners activates intracellular signaling elements, which stimulate peripheral gustatory nerves and, in turn, brain gustatory pathways [9, 10]. The artificial sweeteners weaken a natural predictive relationship between sweet taste and the calorie intake during eating.

One of the main factors for the regulation of the energy homeostasis is ghrelin. It can be found as two isoforms in the circulation: acylated ghrelin (AG) and unacylated ghrelin (UAG). Acylation is catalyzed by the enzyme ghrelin O-acyl transferase. The appetite-stimulating function of AG was identified secondary to its effect on the growth hormone release from somatotroph cells of the anterior pituitary [11]; however, AG is the first known peripheral hormone to display orexigenic effects through its action on the hypothalamic appetite-regulating pathways [12]. AG activates NPY/AgRP neurons [13] of the hypothalamic arcuate nucleus through its receptor, GHS-R1a, promoting production and secretion of their orexigenic neuropeptides to suppress pro-opiomelanocortin neuronal activity while stimulating food intake. There is evidence to show that ghrelin is important in the short-term regulation of appetite and energy balance. The pre-prandial

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rise and post-prandial fall in plasma ghrelin levels support the hypothesis that ghrelin acts as an initiator signal for meal consumption in humans [14]. Ghrelin also appears to be involved in the regulation of long-term energy homeostasis [14]. This peptide hormone has been described as the peripheral counterpart of insulin and leptin, and adenosine monophosphate-activated protein kinase also appears to be involved in its peripheral metabolic effects [15]. Ghrelin reduces the use of fat as a metabolic fuel and promotes an increase in adipose tissue and body weight [16]. Circulating ghrelin induces abdominal obesity, independently of its central orexigenic activity, via GHS-R-dependent lipid accumulation in fat depots [17].

Long considered to be an inert degradation product of AG, UAG (or des-acyl ghrelin) nowadays emerges as an important hormone, apart from the other proghrelin-derived peptides, AG and obestatin. UAG amounts to 80-90% of the circulating orexigenic hormone ghrelin. UAG appears to have its own receptor, and it can share this receptor with AG, under experimental conditions at least. An increasing number of studies suggest that UAG can act as a potent functional inhibitor of ghrelin. It can even strongly suppress ghrelin production in obese human diabetic subjects [18]. Moreover, UAG can improve postprandial glycemia, especially in those subjects in whom preprandial acylated ghrelin levels are high, which makes UAG, or UAG-analogs strong potential candidates for the development of drugs for the treatment of metabolic disorders or other conditions in which elevated AG/UAG ratios occur, such as diabetes, obesity and Prader-Willi syndrome [19].

Therefore, the aim of this study was to track changes in UAG in plasma of experimental rats and to compare them with some morphological and metabolic parameters.

EXPERIMENTAL

Animal models

Male albino Wistar rats weighing circa 250 g were kept in the accredited Vivarium at the Medical Faculty, Trakia University at 25±1°C with a photoperiod light/dark of 12/12 hours and free access to water and food. The standard diet was composed of starch - 50%, protein - 20%, fat - 3%, cellulose - 5%, standard vitamin and mineral mix. After adaptation (one week) an 8-week treatment of the 28 male rats with drinking water (control Group C, n = 7), 15% fructose solution (Group F, n = 7), 10% sucrose solution (Group S, n = 7), and 0.3% aspartame solution (Group A, n = 7) was carried out. Food intake was recorded daily and animal weight was monitored at the end of the 2nd, 4th, 6th and 8th week. The containers with water and sweet solutions were supplemented every two days, once a week the amount consumed by all groups was calculated and the average intake of solutions in g/100 g body weight per day for the experimental groups was determined.

At the end of the experiment the rats were fasted overnight and anesthetized with a mixture of ketamine-xylazine (90 and 10 mg/kg respectively, i.p.). Blood samples were collected for serum and plasma separation by abdominal aorta puncture. Depots of retroperitoneal, mesenteric (visceral) and subcutaneous adipose tissue of the back were removed and weighed immediately after dissection in order to avoid weight loss by evaporation. Fat depots were measured in mg/100 g body weight.

The experiment was conducted in compliance with the requirements of both the national legislation and the European Directive 2010/63/EU of 22.09.2010 on the protection of animals used for scientific purposes.

Blood collection and biochemical analyses

In sera from venous blood collected from the tail vein at the beginning and from the abdominal aorta (under ketamine-xylazine anesthesia) at the end of the experimental period glucose, lipid parameters (triglycerides, total cholesterol, HDL- and LDL-cholesterol), and the levels of uric acid, ALT and AST were examined using an automatic analyzer Mindray BS300.

Blood collection and measurement of unacylated ghrelin in plasma

Blood samples from the abdominal aorta were collected in tubes containing EDTA. Samples were centrifuged at 3,500 rpm for 10 minutes at +4°C and then supernatants were transferred into separate tubes. Samples were stored at -20°C until analysis. Levels of plasma unacylated ghrelin were assessed using the BioVendor Rat Unacylated Ghrelin ELISA kit, based on a double-antibody sandwich technique (BioVendor - Laboratorni medicina, Czech Republik) according to the manufacturer's instructions. The assay sensitivity was 8 pg/ml.

Statistical analysis

The results were analyzed by Student's t-test on Statistica v.12 (StatSoft, Inc.). Correlation analysis with determination of correlation coefficient r was also performed. Differences were considered significant if $p<0.05$.

RESULTS AND DISCUSSION

Following the changes in body weight of the control and experimental groups of rats, it was observed that all experimental groups had higher mean body weight at the end of the 8-week period than the controls (Fig. 1). However, only the animals overloaded with sucrose had significantly higher mean body weight than controls ($p<0.05$).

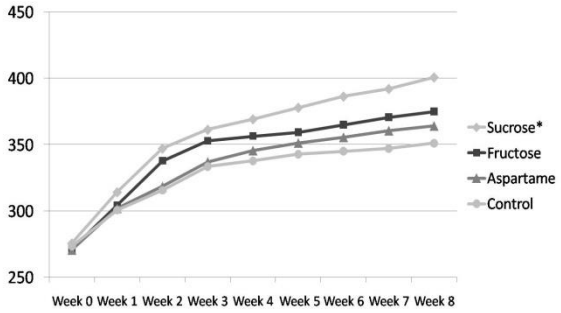

Fig. 1. Dynamics in group mean weight (g) for eight week period, *p < 0.05.

Moreover, rats from Group S elicited greater body mass gain than rats from the other experimental groups. This could be due to the presence of both glucose and fructose derived from hydrolysis of sucrose. Glucose is metabolized widely in the body for energy, glycogen storage or synthesis of fatty acids, while fructose is metabolized mainly in the liver in *de novo* lipogenesis of fatty acids and TAG synthesis. It is noteworthy that, based on the differences in the metabolic pathways for fructose and glucose, fructose consumption gives less satiety, leads to increased food intake and body mass gain compared to glucose. Fructose, unlike glucose does not stimulate insulin secretion. Low levels of insulin in a high-fructose diet could explain the increased food intake [20, 21]. Similar changes in body weight after fructose, sucrose and aspartame consumption found several authors [6, 22, 23].

Increase of the mean retroperitoneal and mesenteric fat deposits was also observed in the following order: Group F > Group S > Group A > Group C, although the differences were not significant in most cases besides the mesenteric fat depot weight of Group F vs. Group C (Table 1). An increase in visceral fat depots in fructose fed animals found Stanhope and Havel [4].

Table 1. The main biochemical and morphological parameters for the four experimental groups of rats. Data are presented as mean value ± SD

Parameter	Control (C)	Fructose (F)	Sucrose (S)	Aspartame (A)
Serum glucose (mM)	5.43 ± 0.75	9.76 ± 2.48*	7.04 ± 0.77*	4.97 ± 1.08
Serum total cholesterol (mM)	1.75 ± 0.12	2.1 ± 0.42	1.95 ± 0.17*	1.79 ± 0.09
Serum HDL-cholesterol (mM)	0.5 ± 0.08	0.39 ± 0.05*	0.66 ± 0.04*	0.69 ± 0.03*
Serum LDL-cholesterol (mM)	0.73 ± 0.07	0.85 ± 0.04*	0.88 ± 0.05*	0.77 ± 0.04
Serum TAG (mM)	0.72 ± 0.24	1.91 ± 0.87*	1.14 ± 0.22*	0.9 ± 0.28
Serum UA (μmol/L)	17.43 ± 5.25	76.85 ± 26.49*	31.86 ± 12.96*	21.57 ± 12.2
AST (U/L)	117.57 ± 19.18	162.86 ± 34.28*	171.71 ± 32.98*	119.00 ± 10.26
ALT (U/L)	41.14 ± 12.21	60.57 ± 15.25*	51.43 ± 7.69	45.86 ± 13.37
Retroperitoneal fat depot weight (mg/100g bw)	815.9 ± 240.15	1137.8 ± 332.24	1082.78 ± 389.75	1035.37 ± 351.75
Mesenteric fat depot weight (mg/100g bw)	1075.64 ± 254.42	1763.45 ± 709.98*	1572.67 ± 602.58	1112.05 ± 492.48
Subcutaneous posterior fat depot weight (mg/100gbw)	124.63 ± 53.13	154.0 ± 61.47	208.72 ± 126.16	163.69 ± 39.83

*p < 0.05 compared with the control group

Bursać et al. [24] analyzed the effects of 9-week consumption of 60% fructose solution on dyslipidemia, insulin and leptin sensitivity, and adipose tissue histology in male Wistar rats. The total body mass of fructose-fed rats was not changed, but the mass of visceral omental adipose tissue, as well as the relative ratio of visceral omental fat mass to total body mass was increased by fructose diet, pointing to visceral adiposity. Their results also showed elevated triglycerides and hypothalamic leptin resistance accompanied by stimulated glucocorticoid signaling and NPY mRNA elevation. Fructose consumption shifted the balance between glucocorticoid receptor and adipogenic transcriptional factors (PPARγ, SREBP-1 and lipin-1) in favor of adipogenesis judged by distinctly separated populations of small adipocytes observed in this tissue. They concluded that high-fructose-diet-induced alterations of glucocorticoid signaling in both hypothalamus and adipose tissue result in enhanced adipogenesis, possibly serving as an adaptation to energy excess in order to limit deposition of fat in nonadipose tissues, which are the key events leading to the development of insulin resistance and type 2 diabetes.

Increased hepatic *de novo* lipogenesis induced by fructose is another possible mechanism which promotes obesity [25]. In healthy volunteers there are data of reduced insulin sensitivity of the liver due to high consumption of fructose [26]. A dose-dependent increase in the fat content of the liver has been observed only after several weeks on a diet rich in fructose [27]. In our study body weight gain reflected the amount of food consumed and was the highest in rats given sucrose, followed by the fructose and aspartame group of rats, which indicate that the non-caloric sweetener aspartame may also increase food intake and cause an increase in body weight. Martinez et al. [6] showed that rats who drank water with aspartame and sucralose were fatter than both control and sucrose groups, in spite of the fact that total caloric intake in the sucrose group was higher than in both groups with artificial sweeteners.

The results of human studies show that consumption of artificial sweeteners in beverages at least daily was associated with significantly greater risk of select metabolic syndrome components (36% greater) and type 2 diabetes (67% greater) [28]. Body mass gain and overeating after consumption of products containing artificial sweeteners can be explained by the hypothesis that artificial sweeteners disrupt the body's natural ability to predict the caloric contents of food on the basis of sweet taste, which leads to greater body mass gain through increased food intake during the next meal to compensate the energy deficit [29]. Sweet tastes are known to evoke numerous physiological responses that help to maintain energy homeostasis by signaling the imminent arrival of nutrients in the gut and by facilitating the absorption and utilization of energy contained in food. This failure to anticipate calories and sugar appropriately when they do arrive could ultimately lead to the negative health consequences associated with artificial sweeteners, by impairing the ability of sweetness to predict the arrival of energy in the gut accurately, thereby reducing the efficient utilization of that energy and perhaps weakening the cascade of events that initiate satiety.

Table 1 shows that consumption of sweeteners causes serious disturbances in the lipid profile expressed in significant increased total (Group S) and LDL-cholesterol (Group F and S), TAG (Group F and S), accompanied by impaired glucose homeostasis (Group F and S). In accordance with our results other authors indicate insulin resistance as a major cause of impaired glucose tolerance [4,30,31].

Also in Table 1 are presented the significant elevations of uric acid and AST in Group F and S, and ALT in Group F, compared with the control group. Increased uric acid is considered a minor criterion in the definition of metabolic syndrome and explains the higher incidence of gout, renal calculosis and type 2 diabetes in those patients due to the creation of pre-receptor insulin resistance. Uric acid inhibits the synthesis of the vasodilator nitric oxide and is considered one of the causes of fructose-induced hypertension. According to Douard and Ferraris [32] for this hypertension contribute expression of the glucose transporter protein (GLUT 5), increased intestinal salt reabsorption and reduced renal salt excretion.

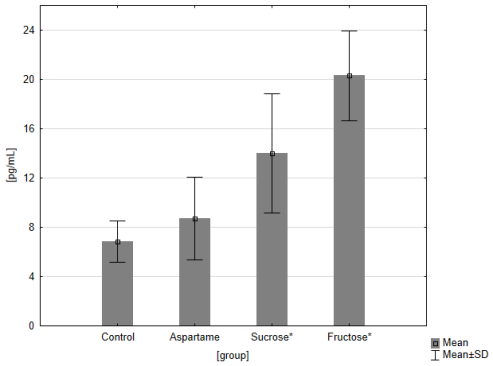

Fig. 2. Levels of plasma UAG at the end of the study period (Mean ± SD); *p < 0.05 compared with the control group.

ALT is an important enzyme found predominately in the liver. Elevated ALT levels correlate strongly with non-alcoholic fatty liver disease and other liver diseases, because significantly elevated activity of ALT in serum

reflects hepatocyte damage. ALT is a more specific indicator of liver inflammation than AST, as AST may also be elevated in diseases affecting other organs.

In our experiment the animals overloaded with sweeteners had higher mean UAG levels than controls in the following order: Group F > Group S > Group A > Group C. Fig. 2 shows significant increase in UAG levels in Group F and Group S. After consumption of fructose (that gives less satiety) or sucrose activation of ghrelin production and AMP-activated protein kinase (AMPK) participation in its peripheral metabolic effects has been described [15]. Kola and Korbonits summarize data about intracellular mechanisms of the appetite-inducing effect of ghrelin in the hypothalamus: AMPK is a key metabolic enzyme involved in appetite regulation. Calmodulin kinase 2 has been identified as an upstream kinase of AMPK and a key mediator in the effect of ghrelin on AMPK activity. The fatty acid pathway, hypothalamic mitochondrial respiration, and uncoupling protein 2 have been outlined as downstream targets of AMPK and mediators of ghrelin's appetite stimulating effect. The increase of UAG levels in Group A, although not significant, may be due to a weaker natural relationship between sweet taste and caloric intake after aspartame consumption [29].

Positive correlations were found between plasma UAG levels and several biochemical parameters: serum glucose ($r = 0.65$; $p = 0.0002$), total cholesterol ($r = 0.46$; $p = 0.01$), LDL-cholesterol ($r = 0.55$; $p = 0.002$), serum triglycerides ($r = 0.47$; $p = 0.01$), uric acid ($r = 0.67$; $p = 0.0001$), AST ($r = 0.5$; $p = 0.006$), ALT ($r = 0.22$; $p = 0.01$), as well as between UAG levels and mesenteric fat depot weights ($r = 0.48$; $p = 0.01$). Negative correlation was observed between UAG and HDL-cholesterol levels ($r = -0.43$; $p = 0.02$). The observed correlations suggest some important links between levels of UAG and the general metabolic parameters. Unfortunately, lack of relevant data from similar experiments in literature limits available resources for discussion.

CONCLUSIONS

The relative elevation of unacylated ghrelin after fructose ingestion in the present study suggests that a failure of fructose to suppress ghrelin could contribute to decreased satiety and increased food intake during long-term fructose consumption.

The development of morphological and metabolic disturbances and an increase of UAG in experimental rats after 8-week consumption of fructose, sucrose and aspartame demonstrates the need to limit the intake of these sweeteners in humans as an opportunity to reduce the current global epidemic of obesity.

Further studies need to elucidate the physiological role of ghrelin in the regulation of food intake. It is possible that detailed understanding of the mechanisms, involved in this process, can lead to the development of therapeutic strategies for some serious nutritional disorders.

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НЕАЦИЛИРАН ГРЕЛИН В ПЛАЗМАТА НА ПЛЪХОВЕ ВИСТАР СЛЕД КОНСУМАЦИЯ НА ФРУКТОЗА, ЗАХАРОЗА И АСПАРТАМ

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(Резюме)

Неацилираният грелин (НАГ) съставлява 80-90% от циркулиращия орексигенен хормон грелин. Проучвания показват, че и двете форми - ацил грелин (АГ) и НАГ могат да медиират периферни биологични функции и НАГ може да действа като мощен функционален инхибитор на грелина. Целта на настоящото изследване бе да се проследят промените в НАГ в плазмата на контролна (К) и три експериментални (фруктозна, захарозна и аспартамна) групи плъхове и да се сравнят с някои морфологични и метаболитни показатели. Проведе се 8 седмично проследяване на 28 мъжки плъхове Вистар (по 7 в група), получаващи питейна вода (група К), 15% фруктозен (група Ф), 10% захарозен (групата З) и 0.3% аспартамен разтвор (група А). Установи се увеличение на средната телесна маса в следния ред: захарозна група> фруктозна група> аспартамна група> контролна група. Намери се сигнификантна разлика в теглото на мезентериалната мастна тъкан при група Ф в сравнение с група К. Регистрира се увеличение на НАГ и на основните метаболитни параметри (глюкоза, триглицериди, общ и LDL-холестерол, AST, ALT) при фруктозната и захарозна групи в сравнение с контролите. Промени в някои метаболитни маркери се установиха и в аспартамната група. В заключение, резултатите от настоящото изследване показват, че увеличената консумация на подсладители като захароза, фруктоза и аспартам води до повишаване на неацилирания грелин, което от своя страна може да предизвика промени в регулацията на приема на храна с последващо развитие на затлъстяване, метаболитни нарушения и хронични неинфекциозни заболявания.