Digest Journal of Nanomaterials and Biostructures Vol. 9, No. 4, October - December 2014, p. 1439 - 1449

THE EFFECTS OF CENTRALLY APPLIED GHRELIN ON APPETITE AND METABOLIC PARAMETERS DURING AGING

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The main purpose of this study was to evaluate specifically, ingestive behavior and blood-borne indicators of metabolic status, after daily intracerebroventricular (ICV) ghrelin injections, in rats of different ages. Four age ranges were tested: peripubertal (~38 days), young (~2 months), adult (~7 months) and middle-aged (~11 months). Multiple variables were measured, including body weight (BW), food and water intake (F,WI), and terminal blood levels of triglycerides (Tg), cholesterol (Chol), free fatty acids (FFA) and glucose (Glu). Five daily ICV injections of ghrelin or saline were administered (n = 8/group, 0.15 nmol of ghrelin in 5 μL) to rats of different ages. After 5 days of treatment, ICV ghrelin resulted in an increased ($p<0.05$) absolute and relative BW, FI, WI and in elevated blood levels of Chol, Tg, and FFA as well, while blood Glu levels were decreased ($p<0.05$) within each of the four age-matched groups. Differences ($p<0.05$) in ghrelin effects over the ages included the following: relative FI was higher in 2 younger groups vs 2 older ones. Relative WI was higher in 2 younger groups vs 2 older ones. Tg, Chol and FFA in the oldest rats, both control and treated, were higher than corresponding values at the other 3 ages. The youngest age group had the lowest Glu after ghrelin.

(Received September 8, 2014; Accepted October 31, 2014)

Keywords: Ghrelin, appetite, Metabolic parameters, Rats, Ageing

1. Introduction

Proper nutrition is essential for health preservation and longevity. Aging is associated with a reduction in appetite and decline in energy intake of approximately 1% per year (1). Aging is correlated with a reduced ability to keep the energy homeostasis, in response to physiological and environmental disturbances (2, 3).

During the aging process, the first changes are metabolic (catabolic processes dominate), leading to gradual reduction in appetite and food intake, described as "anorexia of aging". The causes of such anorexia are mainly unknown and possibly multi-factorial. This anorexia may predispose pathological weight loss and malnutrition (1, 4). However, the key indicator in elderly appears to be the loss of appetite (5). The age-related decrease of food intake, also observed in laboratory animals, has been regarded as normal due to diminished energy demands (6).

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Ghrelin is an endogenous 28-amino-acid peptide with an acylated serine residue at position 3. This unique side chain appears to be the essential for many of its biological effects, including stimulation of appetite and secretion of growth hormone (GH) (7, 8). In rats, intracerebroventricular (ICV), intravenous or subcutaneous administration of ghrelin stimulated food intake and decreased energy expenditure, thus facilitating weight gain (9). In humans, intravenous ghrelin administration increased appetite and stimulated food intake by 28% as an average (10). Ghrelin blood levels increased during the fasting, before the onset of meals, and they decreased within 30 min after feeding, suggesting that ghrelin has served as a signal for anticipating the food intake or meal initiation, and what perhaps, caused the awareness of incoming nutrients (11, 12). Plasma ghrelin concentration is low in obese people and high in lean people, further indicating that plasma ghrelin concentration is inversely proportional to body mass index (13, 14).

It is generally accepted that appetite is controlled by the central nervous system (CNS), and that feeding behavior is regulated by complex mechanisms, particularly in the hypothalamic arcuate nucleus (ARC) (15). In ARC, ghrelin promotes the production and secretion of neuropeptide Y (NPY), agouti-related peptides (AgRP), orexin release and inhibits pro-opiomelanocortin (POMC) neurons. Ghrelin, also induces the c-fos expression, as a marker of neuronal activation in the hypothalamus (16).

Recent studies suggest that ICV applied ghrelin increases fractal complexity and textural disorder of exocrine pancreas tissue in rats of different ages. These findings indicate that central ghrelin has significant morphological effects on adrenal gland and exocrine pancreas (17).

Ghrelin levels as reported, are to be decreased in elderly people (18). Rodent studies gave the inconclusive results. In contrast to data related to humans, plasma ghrelin concentrations and stomach ghrelin contents in aged Fisher-344 rats (25 months) are significantly higher than in young ones (19). Serum ghrelin concentrations did not change with age in C57BL/6J male mice (20).

Many animal studies with ghrelin use pharmacological doses of the peptide, often with a single, brief exposure. In order to assess peptide effects on energy homeostasis, we have utilized a very low dose of ghrelin which we successfully used in our prior experiments (21, 22). Our previous study has shown that repetitive ICV administration of low doses of ghrelin, in young rats, increased consummatory behavior and BW without affecting fecal mass or urine volume (22). Also, food and water consumption increased during the first 2 h immediately after ICV ghrelin injections in young rats (21).

The present study was specifically designed to investigate orexigenic effects of centrally applied ghrelin in rats over a wide age spectrum, and to measure multiple variables related to appetite and energy metabolism. This included daily monitoring of body weight (BW), food and water intake and terminal blood levels of triglycerides (Tg), cholesterol (Chol), free fatty acids (FFA) and glucose (Glu), at the end of the 5-day treatment. Such data should provide an overview of the peptide's central metabolic effects over a large spectrum of the rats' life span. Data from our study may reduce inter-laboratory differences related to dose-response, time action issues and the protocol variability as well.

2. Experimental

Ethical approval. All experimental protocols were approved by the Local Animal Care Committee (School of Medicine, University of Belgrade) and conformed to the ethical terms of reference stated in the United States NIH guidelines [Guide for the Care and Use of Laboratory Animals (1985), DHEW Publication no. (NIH) 85-23: Office of Science and Health Reports, DRR/NIH, Bethesda, MD].

Animals. Experiments were performed on Wistar male rats of different ages: peripubertal (38 days; 153.2 ± 9.0 g BW), young (2 months; 221.3 ± 9.0 g BW), adult (7 months, 469.2 ± 16.5 g BW) and middle-aged (11 months; 676.8 ± 14.9 g BW). All rats were bred at the Institute of Biomedical Research "Galenika" in Belgrade. They were kept in individual metabolic cages under a 12:12 h light–dark cycle, at 22 ± 2 °C, and were accustomed to daily handling. They had a balanced diet for laboratory rats, which contained 20% proteins, 50% corn starch, 10% sucrose,

10% corn oil, 5% cellulose, and 5 % vitamins, minerals and antioxidants (prepared by "D. D. Veterinary Institute "Subotica", Subotica, Serbia). Food and water were available to the rats *ad libitum*.

Animal preparation. Surgical procedures were performed under pentobarbital anesthesia (Thiopental-Sodium, 45 mg/kg, i.p., Rotexmedica, Trittau, Germany). The rats were implanted with a headset, later used for ICV injections. A minimum recovery time of 5 days was permitted before the onset of experiments. The headset consisted of the silastic-sealed 20-gauge cannula (23), implanted into a lateral cerebral ventricle, 1 mm posterior and 1.5 mm lateral to bregma, and 3 mm below the cortical surface. A small stainless steel anchor screw was placed at a remote site on the skull. Cannula and screw were cemented to the skull by dental acrylic (Simgal; "Galenika", Belgrade, Serbia). Following surgery, the animals received a single dose of buprenorphin s.c. 0.28 mg/kg (Buprenex; Reckitt Benckiser Healthcare, Slough, UK). The placement of cannula was confirmed at the end of the experiment by 5 μl injection of Trypan blue dye (23, 24).

Animal treatment. After the recovery period, the animals of the same age were randomly divided in two groups, with eight rats in each. The first group was treated ICV with 0.15 nmol of rat ghrelin (Global Peptide, USA) dissolved in 5 μL of PBS (phosphate buffered saline) every 24 h in 5 consecutive days. Ghrelin dose used in this experiment was based on our previous studies related to ICV ghrelin actions (21; 25). The second group was a control group, with rats treated in the same manner, but injected with 5 μL of solvent only. The ICV injections were given at 10:00 a.m. During the treatment, BW, food and water intake, as well as feces elimination and urine excretion, were obtained daily, just before the next ghrelin or saline injection. All animals were killed by decapitation 1 hour after the last ICV injection, under deep pentobarbital anesthesia (Thiopental – Sodium, 45 mg/ kg, i.p.).

Biochemical assays. Blood samples were collected from each animal immediately after decapitation. Plasma and serum samples were obtained and stored at − 20 °C until assayed. Biochemical blood analyses (Tg, Chol and Glu) were performed by using the peroxidase active principle (PAP) methods. Plasma concentration of FFA was determined by using Randox NEFA test kit (Randox Laboratories, Crumlin, UK), scaled for the use by the colorimetric method.

Statistical analysis. All data are presented as group means ± standard deviation of the mean (SD) for 8 animals per group. Body weights at the beginning (BW_1) and at the end (BW_2) of the experiments were recorded for each rat, and the difference between them was calculated as $BW_2 – BW_1$. This body weight gain (ΔBW) was also calculated as relative to initial body weight (rΔBW). Food and water intake are expressed in absolute values and also as relative to BW_1. Food and water intake are presented as the average 24 hour intake for each rat of different age, and average per group, over the 5-day period.

Statistical data analysis was performed by Student's t-test, Mann-Whitney (U test) and Kruscal-Wallisn, using the SPSS (Statistical Package for IBM PC), 12th edition. Probability values of 5% or less were considered as statistically significant.

3. Results

Body weight, food and water intakes

The influence of 5 daily ICV ghrelin injections on BW, ΔBW and rΔBW in rats of different ages are summarized in Table 1. Central ghrelin treatment significantly increased ($p<0.05$) ΔBW and rΔBW in rats of all ages. Compared with the age-matched controls, the increases of rΔBW were: 30.5% in peripubertal, 13.8% in young, 14.7% in adult and 14.6% in middle-aged rats ($p<0.05$). Among ghrelin treated groups, peripubertal rats had significantly increased ($p<0.05$) rΔBW, compared to other three ages.

Table 1. The effects of ICV ghrelin injections on body weight in rats of different ages

Rats	Group	BW_1 (g)	BW_2 (g)	ΔBW (g)	rΔBW (%)
Peripubertal	Control	163.4 ± 10.0	193.8 ± 3.2	32.4 ± 4.5	20.8 ± 1.6
	Ghrelin	164.6 ± 9.3	210.9 ± 3.3 * (+ 8.3%)	47.8 ± 8.1 * (+ 47,5%)	30.5 ± 1.4 * #
Young	Control	231.5 ± 9.1	245.5 ± 3.2	16.0 ± 1.0	7.3 ± 0.5
	Ghrelin	233.8 ± 10.2	265.4 ± 3.8 * (+ 8.1%)	31.4 ± 7.9 * (+ 96.5%)	13.8 ± 1.4 *
Adult	Control	471.4 ± 15.5	520.4 ± 14.5	44.1 ± 2.1	9.7 ± 1.4
	Ghrelin	480.1 ± 13.3	546.9 ± 14.7 (+5.1%)	68.0 ± 2.6 * (+ 54.3%)	14.7 ± 1.7 *
Middle-aged	Control	677.0 ± 16.8	742.0 ± 17.2	58.1 ± 1.9	9.1 ± 1.3
	Ghrelin	688.4 ± 17.9	785.8 ± 16.5 (+ 5.9%)	95.6 ± 2.3* (+ 64.6%)	14.6 ± 2.1*

BW_1 – body weight before the first ICV ghrelin injection; BW_2 – body weight after the last ICV ghrelin injection; ΔBW – body weight gain (BW_2 - BW_1); rΔBW – body weight gain relative to BW_1. The values are the means ± SD; n=8 per group; * $p<0.05$ vs. control. # $p<0.05$ vs. ghrelin older ages

Data summarizing the effects of repetitive ICV ghrelin administration on daily average food intake (FI) and food intake relative to BW_1 (rFI) are shown in Table 2. Central ghrelin significantly increased ($p<0.05$) FI and rFI, in rats of all ages, compared to age-matched controls. ICV ghrelin increased rFI in peripubertal, young, adult and middle-aged rats by 7.8%, 8.9%, 5.0% and 4.6%, respectively. Peripubertal and young ghrelin-treated rats had significantly higher ($p <0.05$) rFI in relation to adult and middle-aged rats.

Table 2. The effects of ICV ghrelin injections on daily average food and water intake in rats of different ages

Rats	Group	Food intake (g)	Relative food intake (%)	Water intake (mL)	Relative water intake (%)
Peripubertal	Control	11.5 ± 0.4	6.9 ± 0.3 #	12.5 ± 0.4	7.2 ± 0.5 #
	Ghrelin	14.8 ± 0.3 * (+ 11.8%)	7.8 ± 0.2 * #	13.4 ± 0.2 * (+ 7.2%)	8.1 ± 0.3 * #
Young	Control	18.3 ± 0.4	7.7 ± 0.3 #	16.9 ± 0.3	7.5 ± 0.2 #
	Ghrelin	21.1 ± 0.5 * (+ 15.6%)	8.9 ± 0.3 * #	19.4 ± 0.4 * (+ 14.7%)	8.5 ± 0.5 * #
Adult	Control	21.4 ± 0.3	4.2 ± 0.2	25.8 ± 0.1	4.5 ± 0.1
	Ghrelin	23.1 ± 0.3 * (+ 8.1%)	5.0 ± 0.2 *	27.8 ± 0.2 * (+ 7.8%)	5.1 ± 0.3 *
Middle-aged	Control	27.1 ± 0.1	4.1 ± 0.1	28.6 ± 0.7	4.0 ± 0.1
	Ghrelin	29.3 ± 0.2 * (+ 8.2%)	4.6 ± 0.1 *	30.8 ± 0.8 * (+ 7.7%)	4.5 ± 0.2 *

Relative food and water intake are intakes relative to BW_1. BW_1 – body weight before the first ICV ghrelin injection; The values are the means ± SD; n=8; * $p<0.05$ vs. control. #$p<0.05$ vs. adult and middle-aged, control and treated rats.

Data summarizing the water intake (WI) and water intake relative to BW_1 (rWI) by ghrelin-treated rats, during the aging process, appears in Table 2. Ghrelin treatment significantly increased ($p<0.05$) WI and rWI, compared to age-matched controls. The increases of rWI were: 8.1% in peripubertal, 8.5% in young, 5.1% in adult and 4.5% in middle-aged rats. Peripubertal and young rats (control and treated) had significantly higher ($p<0.05$) rWI than adult and middle-aged rats.

Urine volume and fecal mass output were stable over 5-day of the experiment and there were no differences ($p>0.05$) between control and treated rats, as well as between the ages (Table 3).

Table 3. The effects of ICV ghrelin injections on average urine and fecal mass output in rats of different ages

Rats	Group	Urine (mL)	Relative urine volume (%)	Fecal mass (g)	Relative fecal mass (%)
Peripubertal	Control	8.2 ± 0.9	5.4 ± 0.6	7.9 ± 0.9	5.3 ± 1.0
	Ghrelin	8.5 ± 0.8 (+ 3.6%)	5.5 ± 0.6	8.2 ± 0.8 (+ 3.8%)	5.5 ± 0.8
Young	Control	8.8 ± 1.3	4.0 ± 0.7	8.4 ± 1.1	3.8 ± 0.7
	Ghrelin	9.1 ± 1.0 (+ 3.4%)	4.3 ± 0.7	8.7 ± 1.2 (+ 3.6%)	4.0 ± 0.9
Adult	Control	10.5 ± 0.8	2.3 ± 0.2	9.7 ± 0.9	2.0 ± 0.5
	Ghrelin	10.9 ± 1.1 (+ 3.8%)	2.4 ± 0.4	10.0 ± 0.8 (+ 3.0%)	2.2 ± 0.6
Middle-aged	Control	14.0 ± 0.6	2.0 ± 0.1	13.6 ± 0.43	1.9 ± 0.3
	Ghrelin	14.3 ± 0.8 (+ 2.1%)	2.2 ± 0.3	13.9 ± 0.37 (+ 2.2%)	2.1 ± 0.5

Relative urine volume and fecal mass are volume/mass relative to BW_1. BW_1 – body weight before the first ICV ghrelin injection; The values are the means ± SD; n=8;

Biochemical blood parameters after ICV ghrelin

The serum levels of Tg, Chol and FFA in rats of different ages after five consecutive ICV ghrelin injections are shown in Figures 1 and 2. In rats of all ages, ghrelin treatment significantly increased ($p<0.05$) serum levels of Tg, Chol and FFA, compared to their controls. The oldest rats (ghrelin and control) had the highest ($p<0.05$) serum levels of Tg and Chol, compared to younger groups, control and ghrelin (Figure 1). The lowest levels of FFA were in the young rats (both control and ghrelin) versus all other aged groups (both control and ghrelin) (Figure 2).

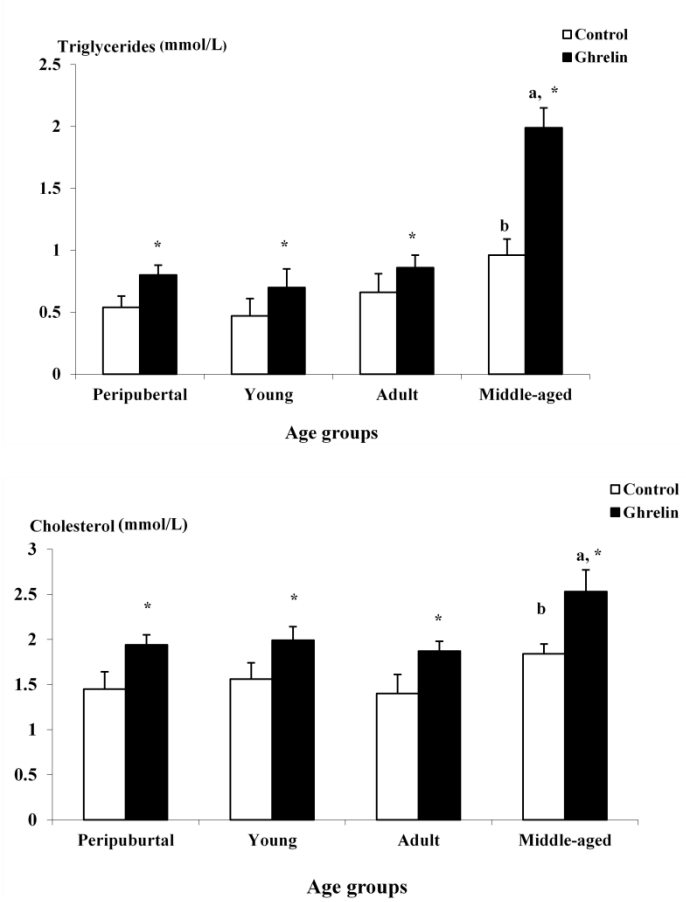

*Fig. 1. The effects of ICV ghrelin injections on serum levels of triglycerides and cholesterol in rats of different ages. All data are expressed as mean ± SD; n=8 per group; * p < 0.05 vs. control; a) p < 0.05 middle aged vs. adult, young and peripubertal ghrelin treated group ; b) p < 0.05 middle aged control vs. adult, young and peripubertal controls*

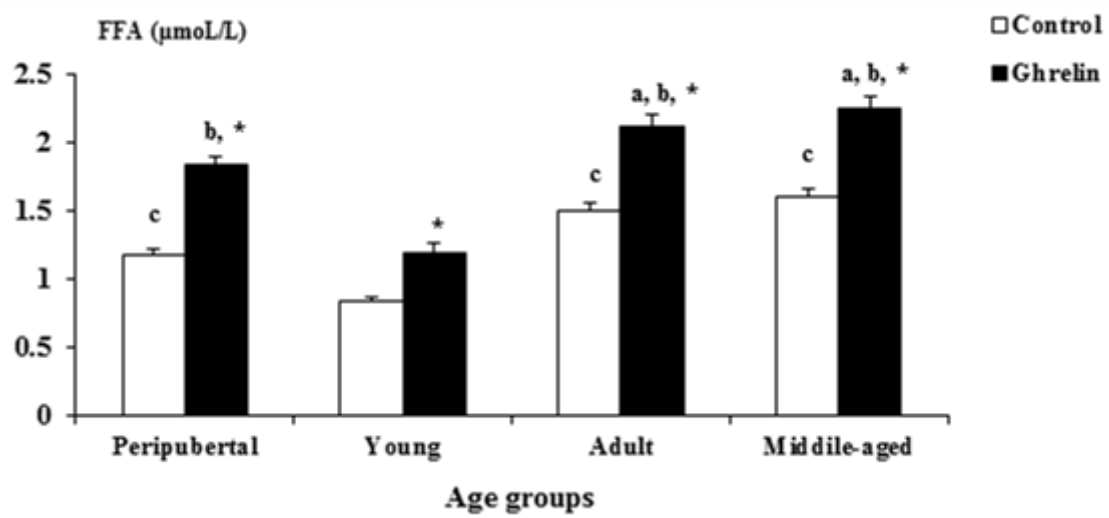

*Fig. 2. The effects of ICV ghrelin injections on serum level of free faty acids in rats of different ages. All data are expressed as mean ± SD; n=8 per group; * p < 0.05 versus control; a) p < 0.05 middle-aged and adult vs. young and peripubertal ghrelin treated rats b) p < 0.05 middle-aged, adult and peripubertal vs. young ghrelin treated rats; c) p < 0.05 middle-aged, adult and peripubertal controls vs. young controls*

Fig. 3 shows the serum levels of Glu in rats during aging, ghrelin or saline treated. In peripubertal, young, adult and middle-aged rats, serum Glu levels were significantly lower ($p<0.05$) by 28.1%, 17.6%, 22.6% and 14.5%, respectively, after ghrelin treatment, compared to corresponding controls. Among ghrelin treated rats, the lowest glucose levels were in the peripubertal age, versus all other ages.

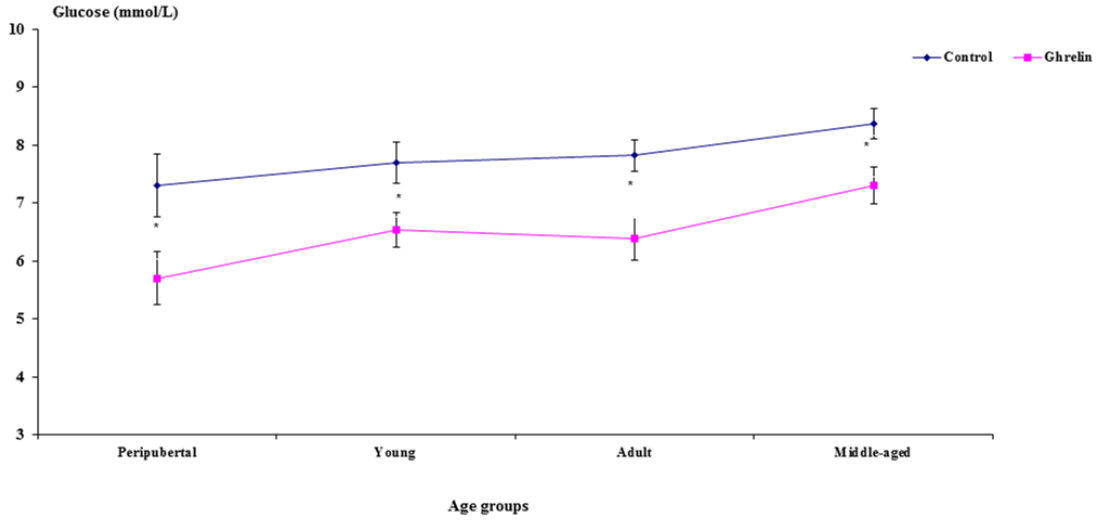

*Fig. 3. Serum concentration of glucose in control and ghrelin-treated rats of different ages. All data are expressed as mean ± SD; n=8 per group, * p < 0.05 vs. control.*

4. Discussion

The present study regarding the ICV ghrelin treatment, at nanomolar doses over a 5-day span, results in consistent stimulatory effects on consummatory behavior. Also ICV ghrelin influences both, lipid and carbohydrate metabolism. These changes occurred in animals ranging at the age from about 38 days to 11 months; i.e. approximately from onset of adulthood to middle/elderly age. Ghrelin exerted identical qualitative effects on the measured parameters across the ages, but quantitative changes appeared concomitant with the aging process. A comparison of these results with corresponding literature data are as follows:

The present study demonstrated that ICV ghrelin treatment, of all age groups, increased body weight compared to the age matched saline controls. The relative weight gain was the greatest in the youngest rats.

Kamegai et al. (26) noted that ICV ghrelin, which was applied every 12 hrs for 72 hrs, increased the body weight significantly. Tschop et al. (27), showed that an ICV ghrelin infusion evoked a significant and dose-dependent increase in weight and fat content. Our previous studies showed that the weight gain after ICV ghrelin was ascribed to increase retroperitoneal and epididymal fat deposits (21, 22).

In the present study ICV ghrelin increased absolute and relative food intake of all four ages vs matched controls. The relative food intake declined in both saline and ghrelin treated rats in the oldest two groups, compared to the younger ones.

Gilg & Lutz (28) reported that multiple i.p. ghrelin injections (15 μg/kg/day over 10 days) increased the food intake in adult rats after four days. On the other hand, 2 hrs after i.v. ghrelin administration, food intake of 27-months old Long Evans rats was ~ 30% lower than in 3-, 12-, and 24-months old rats (29). Akimoto-Takano et al. (30), found that ICV ghrelin application in different doses induced a dose-dependent increase in food ingestion in 4, 11 and 24 months aged Wistar rats.

It is noteworthy that three neuropeptides that stimulate food intake (ghrelin, orexin and NPY) differ with regard to age and dose (30). It is of interest that only ghrelin showed a substantial stimulation in old rats (7). The appetite-stimulating effect of ICV ghrelin was reduced with

advancing age, but it has been still significantly increased compared to orexin and NPY (6, 31, 32). Those results appear to be comparable to the present study where the relative food intake declines after ghrelin in older rats, but it still produces a measureable increase in feeding.

An average daily water intake increased at the all age tested. Both treatments, saline or ghrelin increased WI in a stepwise manner with aging. Comparing the relative water intake (volume related to initial body weight), the two older groups had lower values than two younger ones. Drinking behavior was mainly related to feeding and the increase may be related to prandial drinking due to maintain osmotic homeostasis (22).

It was found that ICV ghrelin markedly suppressed drinking evoked by ICV angiotensin (33). Hashimoto et al. (34, 35) also reported that ICV or peripheral administration of ghrelin markedly inhibits water intake in rats that were deprived of food and water for 24 hours. On the other hand, Mietlicki et al. (36, 37) observed no effect of ICV ghrelin in young rats who were water deprived for 24 hours. Role of ghrelin's resolution in drinking behavior is still unresolved, as mechanisms involved in drinking appear to be highly redundant after water deprivation (24).

Urine volume and fecal output were unchanged in the present experiments. Unchanged fecal output along with increased food intake was consistent with the anabolic effect of ghrelin (38).

The present results also demonstrated increased Tg, Chol and FFA serum levels in ghrelin-treated rats versus respective controls at all ages. The oldest rats, both control and ghrelin, had the highest serum levels of Tg and Chol, compared to the corresponding values at the three younger groups. Theander-Carillo et al. (39) also evaluated the effects of ICV ghelin (2.5 nmol/day x 6 days) and observed a fall in serum Tg and a trend to lower glucose levels. They interpreted these data as increased glucose and Tg uptake into white adipose tissue, increased lipogenesis and reduced lipid oxidation. High serum levels of FFA may be due to increase secretion from hypertrophic adipocytes (40). Perez-Tilve et al. (41) studied the effects of peripherally administered ghrelin (2.5 nmol/day x 7 days) by using the wild-type mice. Under these conditions, ghrelin elevated blood Chol, whereas glucose and Tg were unchanged. All together, the reasons for ghrelin-induced hyperlipidemia likely reflect a centrally-mediated altered lipid turnover within the gut, liver and adipose tissue depots.

The role of ghrelin on blood glucose regulation remains unclear. The present study found that centrally administered ghrelin significantly decreased serum Glu levels by 20% as an average versus controls at all ages. So it could be interpreted as an influence of ghrelin on glucose uptake into lipid stores (21, 27, 39) clearly demonstrated adipogenic effects of ghrelin. Conversely, Kamegai et al. (26) showed that repeated injections of ghrelin into the lateral ventricle of rats during 72 hours, caused no changes in plasma glucose and insulin concentrations, but there was a trend to higher levels.

5. Conclusions

Daily intracerebroventricular injections of subnanomolar doses of ghrelin in 5 consecutive days evoke clear changes in consummatory behavior, carbohydrate and lipid metabolism in rats over all investigated ages. In ghrelin treated rats food intake is increased at all ages. The relative food intake is reduced in adult and middle-aged rats, compared to younger ones. The water ingestion pattern follows a similar food ingestion pattern. Changes in lipid parameters evoked by ghrelin, accompanying by decline in blood glucose levels are reflected as an increased blood cholesterol, triglycerides and free fatty acids. These data and the available literature as well, support the opinion that endogenous ghrelin, both central and peripheral at all ages, is the key regulator of consummatory behavior and carbohydrate/lipid homeostasis.

We hope that understanding the cause of age-related diseases on food intake, may lead to the development of therapeutic approaches that can counteract aging-associated disintegration of energy homeostasis.

Acknowlegment

This work was supported by the Ministry of Education and Science, Government of Republic of Serbia, grants No III 41025.

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