Abstract

Insulin is a hormone and a drug with well-known properties. It is well established that obesity is characterized by hyperinsulinemia and hyperleptinemia. Leptin and ghrelin play a crucial role in the regulation of appetite and energy homeostasis in rodents and humans. The aim of this study was to investigate the effect of insulin and glucose administrated in the third ventricle on leptin and ghrelin expression in the hypothalamus, and to further elucidate the mechanisms of leptin resistance in obese animals. Male Wistar rats were used in the experiment. A nutritional model of obesity was developed based on our previous experience. Then, an intracerebroventricular infusion of insulin and glucose was performed. Immunohistochemical materials were analyzed for leptin and ghrelin positive cells. The results demonstrate an inhibiting effect of both insulin and glucose on leptin in the brain. No effect on ghrelin was detected. These findings suggest a role of insulin in leptin resistance in obesity.

Key words: insulin, glucose, ICV, immunohistochemistry, obesity

Introduction. Leptin and ghrelin are the major regulators of appetite and metabolism [1, 2]. Leptin is an anorexogenic hormone derived from the fat tissue [3], whereas ghrelin is an orexogenic hormone secreted by the stomach cells [4]. These hormones are thought to be the main regulators of appetite...
and metabolism [5]. Leptin levels rapidly fall when there is caloric restriction and weight loss [1]. Plasma ghrelin levels are high when fasting, and decrease when eating [2]. Ghrelin is the hormone that increases appetite, increases body weight and reduces fat oxidation [6]. The main brain regions responsible for the regulation of energy homeostasis are the hypothalamus and the brainstem [7]. The nucleus arcuatus can be considered as a key afferent area for the regulation of energy homeostasis [7]. Obesity is a condition characterized by hyperleptinemia, hyperinsulinemia, and hypeglycemia in rodents and humans [8,9]. Obesity is associated with leptin and insulin resistance. When insulin resistance is accompanied by dysfunction of pancreatic islet beta-cells and subsequently by hyperglycemia, the risk of developing type 2 diabetes significantly increases. Our experimental model of obesity demonstrates the parameters of hyperleptinemia in the experimental rats [10,11]. This is most probably due to the increased fat tissue. In rodents fed a high-fat diet to induce obesity and insulin resistance, islet sizes increase as a result of beta-cell rather than a change in beta-cell size, and new islets do not form [12]. It is established that the high-fat induced obesity is a model of [13]. The metabolic changes after chronic fat intake are associated with the decreased leptin signals to the brain, and the decreased inhibition of appetite [13,14]. There are data in the literature that leptin resistance is a result of low leptin expression in the hypothalamus, and alternated brain-blood barrier [15,16]. Furthermore, SCARPACE et al. [17] show that leptin plays a beneficial role in the development of model of obesity in rats fed by a high-fat diet.

**Materials and methods.** The experiments were carried out according to the “Principles of laboratory animal care” (NIH publication No 85-23, revised 1985), and the rules of Ethical Committee of the Medical University in Sofia.

**Animals.** Male Wistar rats (110 g) were used in the study. After one week of adaptation rats were randomly assigned to two different groups: 1st control group (n = 10), and 2nd experimental group (n = 10). Rats were housed individually in polypropylene boxes with free access to food and water. The animals were maintained in a constant environment (22 ± 2°C) on 12 h light/12 h dark cycle. The 1st group was fed by a standard laboratory chow food, whereas the 2nd group was fed by a high-fat diet (HFD) and chow food in order to induce obesity in rats. Both diets were ad libitum. The experimental nutritional model of obesity lasted for 3 months using our previous experience [10]. Body weight was measured weekly.

**Drugs.** After this period rats were divided in groups for further infusion of insulin/glucose/saline solutions. Glucose solution was ex tempore prepared (1 g dry compound was dissolved in 7.2 ml saline solution; Insulin (Actrapid Penfill) was purchased from Novo Nordisk (3 µl insulin was dissolved in 357 µl saline solution 1:120); Saline solution (0.9% NaCl).
Stereotaxic implantation and drug injection in the nucleus arcuatus of the hypothalamus. Animals were anaesthetized with Ketamine (50 mg/kg i.p.), and placed in a stereotaxic apparatus (Narishige S.I.L.). Stainless steel guide cannulae were positioned vertically in the third ventricle at the following coordinates (AP = −1 mm; L = +1.5 mm; H = 3.0 mm) according to the Stereotaxic Atlas – Pellegrino and Cushman [18]. The cannulae were fixed into position with dental acrylic. After surgery the animals were allowed 7 days recovery period. During this period rats were handled daily. Drugs (3.6 µl for each) were injected over a period of 1 min through an injection cannula connected by polyethylene tubing with a constant rate microsyringe Hamilton (Reno, NV). The injection cannula was left in place for additional 30 s. Control rats were injected with saline solution (3.6 µl). Immediately prior to killing, the animals were injected with 1 µl of 2% fast green dye through the injection cannula. Injection sites were then verified automatically postmortem in 50 µm coronal brain sections cut through the hippocampus. Animals were excluded if cannula placement was outside borders of the third ventricular as depicted in Pellegrino and Cushman or were not symmetrical. Three groups of rats: insulin, glucose, and saline treated animals were studied.

Immunohistochemical methods to determine leptin expression in the hypothalamus in rats with/without obesity. All animals were anaesthetized with Thiopental (40 mg/kg i.p.), and were transcardially perfused with 4% solution of paraformaldehyde for 20–30 min. Immediately after the hypothalamuses were taken and put in a fixation solution for additional 2 h at room temperature. After numerous washes in 0.1 M phosphate buffer, pH 7.4, the material was left for 12 h in 0.01 M phosphate sodium-chloride buffer (PBS), pH 7.2–7.4 in refrigerator at 4°C.

The immunohistochemistry was performed in serial cuts prepared in cold microtome (Reichert-Jung). Every seventh cut was used with 40 µm thickness. The following materials were used: Polyclonal rabbit antibody Ob (Y-20): sc-843, (Santa Cruz); Polyclonal goat antibody Ghrelin (c-18): sc-10368, (Santa Cruz), Secondary antibody – biotinilated anti-rabbit IgG, (Santa Cruz), Avidin-biotin-peroxidase kit ABC, (Vectastain ABC kit, Vector Laboratories), and 3, 3’-Diaminobensidine (DAB), (Sigma).

After the immunohistochemical procedures cuts were put on slides, left to dry at room temperature, and finally covered with Entelan (Merck). All slides were examined by light microscope using computerized analyzer Olympus-CUE2. The immunohistochemical positive cells are highlighted. After marking of all cells, the image analyzer receives information on the number of cells in the test box and their density per unit area.

Results. During the chronic study rats on a HFD were gradually increasing their body weight. At the end of the 3-month nutritional period the average increase in weight was with 50–75 g more compared to the control group. The
results demonstrated the development of nutritional model of obesity in rats fed with HFD.

The intracerebroventricular (ICV) injection of insulin and glucose led to suppression of leptin expression in the hypothalamus in comparison to the saline-treated rats both in the control rats (Figs 1, 2, 3) and obese rats (Figs 4, 5). These results demonstrate that the inhibiting effect of leptin on appetite is directly regulated and suppressed by high insulin and glucose concentrations in the hypothalamus area, more specifically in the nucleus arcuatus.

No ghrelin expression in the hypothalamus was found after insulin and glucose ICV infusion.

Discussion. Obesity is characterized by changes in plasma levels of insulin, glucose and leptin [8, 9]. The evidence suggests that leptin, insulin and glucose regulate appetite and metabolism [20]. There are conflicting data in the literature on the impact of high-fat diet and central effects of insulin and glucose in the regulation of leptin. Not enough data in the literature are available on the central effects of insulin and glucose on the expression of leptin in the hypothalamus (nucleus arcuatus). According to Fujita et al. [19] high levels of insulin and glucose in the brain may lead to leptin resistance and food intake alterations in normal-weight animals (without obesity). The authors speculate that changes in leptin receptors occur after ICV infusion of insulin and glucose [19]. Our study shows that single insulin and glucose infusion modulates the leptin expression in nucleus arcuatus in rats with diet-induced obesity. It is well known that a high-fat diet leads to hyperleptinemia in male rats [11]. Furthermore, we documented that a single ICV insulin and glucose administration reduces the expression of leptin in the nucleus arcuatus in animals without obesity. The low leptin expression in the brain could shed light on the understanding of the leptin resistance in obese animals and humans. Suggestive evidence indicates that nucleus arcuatus plays a central role in regulation of appetite and metabolism [7]. More interestingly, it is established that insulin antagonizes the inhibiting effect of leptin on appetite [7, 20]. Data show that obese people have increased plasma leptin levels but no decreased appetite [1]. Further, obese people suffer from hyperinsulinemia [1]. Taken together these physiological findings led us to the idea to examine the effect of insulin administrated ICV on leptin expression in obese animals. Overall,

Fig. 1. Histomorphological cut of hypothalamus. Leptin expression in the nucleus arcuatus. Control group treated ICV with saline solution. 10×

Fig. 2. Histomorphological cut of hypothalamus. Leptin expression in the nucleus arcuatus. Control group treated ICV with insulin solution. 20×

Fig. 3. Histomorphological cut of hypothalamus. Leptin expression in the nucleus arcuatus. Control group treated ICV with glucose solution. 20×

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Fig. 4. Histomorphological cut of hypothalamus. Leptin expression in the nucleus arcuatus. Experimental group treated ICV with glucose solution. 10×

Fig. 5. Histomorphological cut of hypothalamus. Leptin expression in the nucleus arcuatus. Experimental group treated ICV with insulin solution. 10×

Our results demonstrate that the hyperinsulinemia in the brain of obese animals decreases the leptin expression in the nucleus arcuatus. Moreover, our findings could lead to understanding of the mechanisms of leptin resistance.

REFERENCES

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