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Cannabinoids enhance gastric X/A - like cells activity

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Abstract: It has been reported that cannabinoids may cause overeating in humans and in laboratory animals. Although, endogenous cannabinoids and their receptors (CB1) have been found in the hypothalamus, and recently also in gastrointestinal tract, the precise mechanism of appetite control by cannabinoids remains unknown. Recently, ghrelin - a hormone secreted mainly from the stomach X/A-like cells was proposed to be an appetite stimulating agent. The aim of this study was the evaluation of the influence of a single ip injection of a stable analogue of endogenous cannabinoid - anandamide, R-(+)-methanandamide (2.5 mg/kg) and CP 55,940 (0.25 mg/kg), an exogenous agonist of CB1 receptors, on ghrelin plasma concentration and on ghrelin immunoreactivity in the gastric mucosa of male Wistar rats. Four hours after a single injection of both cannabinoids or vehicle, the animals were anaesthetized and blood was taken from the abdominal aorta to determinate plasma ghrelin concentration by RIA. Subsequently, the animals underwent resection of distal part of stomach. Immunohistochemical study of gastric mucosa, using the EnVision method and specific monoclonal antybodies against ghrelin was performed. The intensity of ghrelin immunoreactivity in X/A-like cells was analyzed using Olympus Cell D image analysis system. The attenuation of ghrelin-immunoreactivity of gastric mucosa, after a single injection of R-(+)-methanandamide and CP 55,940 was accompanied by a significant increase of ghrelin plasma concentration. These results indicate that stimulation of appetite exerted by cannabinoids may be connected with an increase of ghrelin secretion from gastric X/A-like cells.

Key words: Cannabinoids - Ghrelin - X/A-like cells - Rats

Introduction

Marijuana is a well-known recreational drug that has for centuries been prescribed therapeutically in herbal medicine for the treatment of many disorders, including loss of appetite [1]. The identification of the major active constituent of marijuana, Δ^9-THC [2], followed by the biochemical and pharmacological characterization of the cannabinoid receptors [3,4], provided a solid foundation and opened a new perspectives for the study of this neurochemical system [5]. Moreover, the isolation of an endogenous ligand of cannabinoid receptors - anandamide, description of its synthesis and metabolic pathways indicated for the existence of an endocannabinergic system [4,6]. Two cannabinoid receptor types CB1 and CB2 have been cloned [3]. The latter appears to be expressed mainly by immune cells, whereas CB1 receptor is expressed by many central and peripheral nerve cells, including vagal afferent neurons [4,7].

There is support for the notion that plant-derived cannabinoids, as well as endogenous and exogenous cannabinoiods increase food intake [8,9]. Both central and peripheral sites of action may be involved [10]. Recently it has been indicated that the effect of endogenous cannabinoids on appetite is mediated by vagal afferent neurons, suggesting a role in modulating gut - brain signaling [11,12]. The cannabinoid system interacts with several peptides involved in the complex regulation of energy homeostasis such as leptin [13], orexin [14] and ghrelin [15]. Some authors support the hypothesis that an increase of ghrelin release in the peripheral serum is a hunger signal devoted to prevent energy deficit and to activate meal initiation [16,17].

Ghrelin is a hormone recognized as a main endogenous ligand for growth hormone secretagogue receptors (GHS-R), that plays an important role in growth

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hormone release and control of feeding behavior [16,17,18]. GHS-R1a is highly expressed in the hypothalamus and pituitary gland, consistent with the actions of ghrelin on the anterior pituitary, as well as with its influence on the control of appetite, food intake and energy balance [16,18]. Moreover, recently the expression of GHS-R1a was described in rat and human vagal afferent neurons of nodose ganglia [19].

The stomach is the main source of circulating ghrelin levels. Ghrelin expression has been localized in X/A-like cells that account for 20-25% of all endocrine cells in the mucosa [18]. Ghrelin has also been detected in many other organs such as the pancreas, bowel, kidney, placenta, gonads, thyroid, adrenal, lung, pituitary and hypothalamus [20,21,22] and also in many neoplastic tissues [23]. The potential physiological role of ghrelin as an autocrine and paracrine factor in these tissues is still under investigation. The orexigenic action of ghrelin is independent of its GH-releasing activity and is mediated by a specific central network of neurones that is also modulated by leptin [24]. According to this hypothesis ghrelin and leptin might be complementary players of one regulatory system that has developed to inform the central nervous system about the status of energy balance [24,25]. Nevertheless, other data do not support a role for the regulation of circulating ghrelin by leptin levels independently of changes in adiposity, and suggest that the leptin and ghrelin systems for energy homeostasis function independently in healthy human subjects [26].

From the gastrointestinal tract, ghrelin could regulate food intake and energy homeostasis reaching GHS-R in the hypothalamus through the general circulation [16,18]. Within the hypothalamus, ghrelin binds mostly on presynaptic terminals of NPY neurones; it stimulates the activity of arcuate NPY neurones and mimicks the effect of NPY in the paraventricular nucleus of the hypothalamus [27]. Thus ghrelin could represent a novel regulatory circuit controlling appetite and energy homeostasis by stimulating the release of orexigenic peptides and neurotransmitters [25].

In an attempt to evaluate whether orexigenic activity of cannabinoids is connected with ghrelin release, the influence of a single ip injection of a stable analogue of anandamide - R-(+)-methanandamide (2.5 mg/kg) and CP 55,940 (0.25 mg/kg), an exogenous agonist of CB1 receptors, on ghrelin plasma concentration and on ghrelin immunoreactivity in gastric mucosa in male Wistar rats was investigated.

Materials and methods

Animals. The study was performed on thirty, male Wistar rats weighing 160 - 170 g. All animals had free access to standard granulated diet and drinking water. The animals were housed in plastic cages at 22±1°C and constant humidity, with a 12/12 light/dark cycle, beginning at 7 am. The rats were randomly divided into 3 groups with 10 animals in each group: rats injected with R-(+)-methanandamide (2.5 mg/kg) or CP 55,940 (0.25 mg/kg) and a control group injected with vehicle under the same experimental conditions.

Four hours after a single injection of both cannabinoids or a vehicle, the animals were anaesthetized with pentobarbital sodium (50 mg/kg b.wt), their abdomen was opened by midline incision and the blood was taken from the abdominal aorta of each rat for the measurement of ghrelin serum concentration by radioimmunoassay (RIA).

Subsequently, all rats underwent resection of distal part of stomach. The tissues were fixed in Bouin's fluid and were prepared to immunohistochemical investigation.

Determination of ghrelin plasma concentration. The blood, taken from abdominal aorta of each rat, was collected into polypropylene tubes without anticoagulant and was incubated in room temperature until the clot was formed and then centrifuged (2500 × g for 15 min). The supernatant (serum) was removed and stored at -20°C until a consequtive analysis. Ghrelin level was determined by the double-antibody radioimmunoassay technique. The protocol for radioimmunoassay kit is accessible on the web site: www.phoenixpeptide.com.

The immunocytochemical study. The distal part of stomach of each animal was fixed in Bouin's fluid for one day at 4°C. After thorough washing in 0.1 M phosphate buffer (pH=7.4) at 4°C, the tissue was routinely embedded in paraffin, and 5-µm-thick sections were cut. For blocking of the endogenous peroxidase activity Peroxidase Blocking Reagent (Dako Poland) was used over 10 minutes. In these studies a specific antibody against ghrelin (Phoenix) was used. After washing with distilled water and 0.05 M TRIS-HCl pH=7.4, three times for 5 minutes, the sections were incubated with the antibody for 1 hour at room temperature. Then, sections were washed three times in TRIS buffer. Subsequently, the EnVision method was applied, according to the protocol for identification of the immunocytochemical reaction [28]. The sections were counterstained with Mayer's haematoxylin. In the negative control, the specific antibody was omitted in the staining procedure. Positive control was done for specific tissue recommended by the producer. The histological preparations were subjected to analysis, using Olympus B ×50 microscope.

Image analysis. To quantify immunoreactivity of the examined marker, computerized image analysis was performed. Images were captured via video link to an Olympus BX50 microscope at 20 objective magnification, so the tissue fully occupied each field, and was scanned by the computer. Pictures were adjusted for optimal contrast, fixed at the same brightness levels, and saved in a buffering system. Staining was analyzed using Olympus Cell D image analysis computer system as described in details by Postek *et al.* [29]. The average optical density was analyzed for cells expressing ghrelin in both, experimental and control rats. The average optical density is this method measured values range from 0 to 255 where 0 means black and 255 white color.

Ethical issues. All procedures were performed in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Local Ethics Committee in Bialystok.

Statistics. All values were given as mean ± SD. The Mann-Whitney test was used for testing the differences between both groups in the intensity of immunocytochemical reactions. The Student-t test was used for the evaluation of the differences between groups in ghrelin plasma concentrations. The value $p<0.05$ was considered to be significant.

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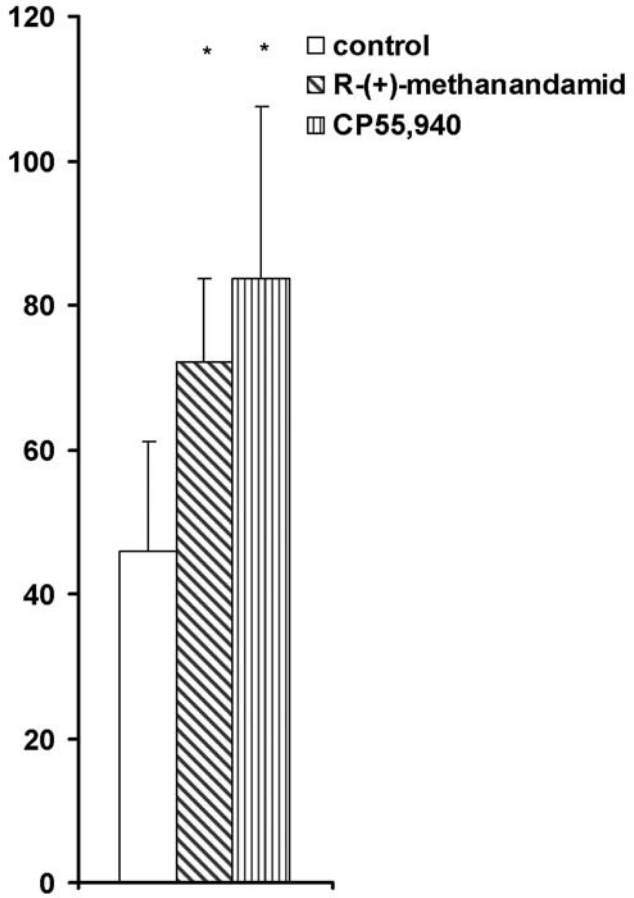

Fig. 1. Ghrelin plasma concentration evaluated 4 hours after a single i.p. injection of R-(+)-Methanandamide (2.5 mg/kg) and CP 55,940 (0.25 mg/kg). Values are means ±SD, *p<0.01 vs control (Student's test).

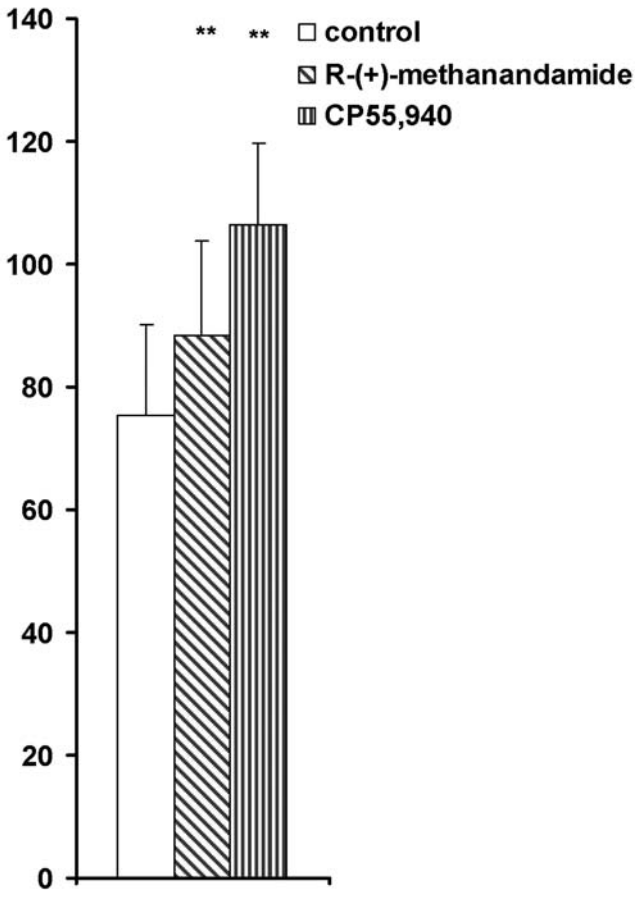

Fig. 2. Average optical density of immunohistochemical staining for ghrelin in X/A-like cells after a single i.p injection of R-(+)-Methanandamide (2.5 mg/kg) and CP 55,940 (0.25 mg/kg). Values are means ±SD, **p<0.001 vs control (Mann-Whitney test).

Results

Radioimmunoassay

Four hours after a single ip injection of R-(+)-methanandamide (2.5 mg/kg) and CP 55,940 (0.25 mg/kg) significant increase of ghrelin plasma concentration (mean values 72.04±11.69 pg/100 µL and 83.7±23.07 pg/100 µL, respectively) as compared to the mean value in the control rats (46.0±15.04 pg/100 µL) was observed (Fig. 1).

Results of histological study

Ghrelin immunoreactive (Ghr-IR) cells were distributed predominantly as single cells, locally in varying number in the deep zone of the gastric mucosa. Ghr-IR cells were most abundant in lower parts of gastric proper glands, while in the neck zone of the glands they were observed sporadically, both in the control (Fig. 3) and in the experimental groups of rats (Fig. 4 and 5). These cells were usually round or oval, sometimes spindle-shaped, seldom irregular with short processes. Their nuclei were usually large, round structures that occupy the central portions of cells. While in the X/A like cells of control rats GH-immunoreactivity appeared as a very strong reaction (Fig. 3), after a single injection of R-(+)-methanandamide (Fig. 4) and CP 55.940 (Fig. 5), GH-immunoreactivity in gastric mucosa was much weaker. The average optical density of immunocytochemical reaction for ghrelin, evaluated by Olympus Soft program, was significantly increased in cannabinoids injected groups of rats in comparison to the control gastric mucosa (Fig. 2).

Discussion

The main finding of the present study is the stimulatory effect of a single ip injection of R-(+)-methanandamide, a stable analogue of endogenous cannabinoid - anandamide, and CP 55,940, a potent, egzogenous agonist of CB1 receptors, on ghrelin secretion from stomach X/A-like cells. The increase of ghrelin plasma concentration after application of R-(+)-methanan-

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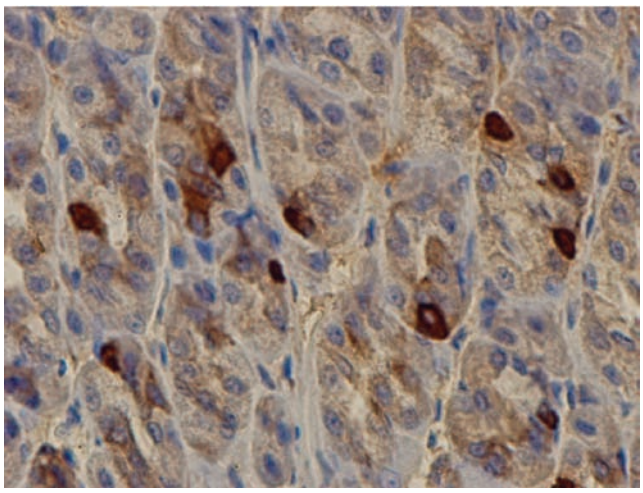

Fig. 3. Light micrograph of gastric mucosa of a control rat. Positive immunohisochemical reaction for ghrelin is observed in X/A-like cells (magnification ×400).

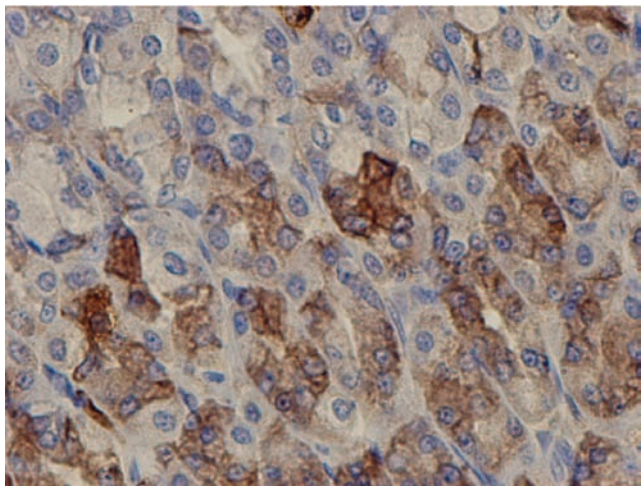

Fig. 4. Light micrograph of gastric mucosa of rat 4 hours after a singl i.p injection of R-(+)-methanandamide (2.5 mg/kg). The attenuation of immunohistochemical reaction for ghrelin is observed in most of X/A-like cells (magnification ×400).

damide and CP 55,940 may partially explain the orexigenic action of cannabinoids.

Our observations are in agreement with an attenuation of ghrelin level after administration of SR141716, a selective CB1 receptors antagonist, reported by Cani et al [15]. They indicated that short-term action of a single injection of SR141716 on appetite is in accordance with the control of ghrelin secretion, a gastrointestinal orexigenic peptide, mainly expressed in the upper part of gastrointestinal tract [15].

The appetite stimulating effect of the Marijuana has long been known [8]. Plant-derived cannabinoid, Δ^9-THC has been shown to stimulate feeding in a variety of animal models [8,30]. Δ^9-THC-induced feeding was reversed by the pretreatment with CB1 receptors selective antagonist, SR141716, that provide a good evidence that this effect is CB1 receptors mediated [15,31]. The hyperphagic action was observed also following administration of endocannabinoids: anandamide and 2-AG [32,33,34]. The increase of food intake was observed after systemic and central injection of endocannabinoids and their action was also reversed by CB1 receptors antagonist [10,15,33]. The involvement of CB1 receptors in the regulation of feeding behavior was confirmed by the administration of the selective CB1 receptor antagonist, SR141716 [15,31]. It has been reported that an acute peripheral and central administration of SR141716 produced an inhibition of food intake in laboratory animals [15].

There is support for the notion that in cannabinoids action regulating feeding behavior central and peripheral mechanisms should be considered. The hypothalamus and its discrete subregions have long been considered to play a key role in integrating the multiple biochemical and behavioral components of feeding and weight regulation [6]. The involvement of the central mechanism in cannabinoids action was confirmed by the observation that cannabinoid activity in the hypothalamus varied according to nutritional status and the expression of feeding behavior [33,35] and also by the finding that cannabinoids administration into hypothalamic nuclei induce eating [32,33].

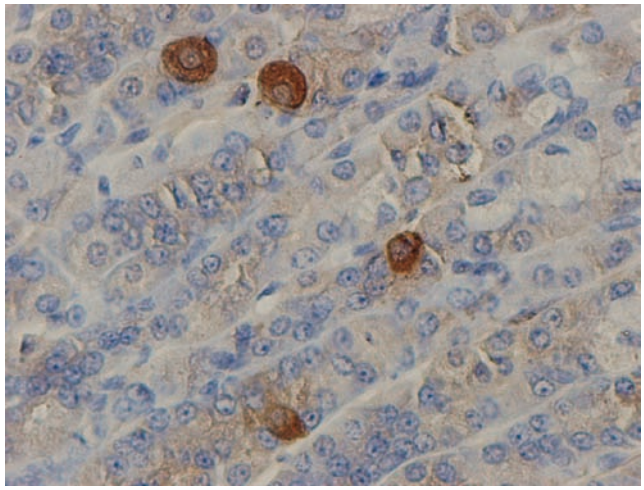

Fig. 5. Light micrograph of gastric mucosa of rat 4 hours after a single i.p injection of CP 55,940 (0.25 mg/kg). The attenuation of immunohistochemical reaction for ghrelin is observed in majority of X/A-like cells (magnification ×400).

However, according to Gomez *et al.* [12] cannabinoids effect on food intake involves predominantly CB1 receptors localized on capsaicin-sensitive sensory terminals present in gut. This hypothesis is based on a lack of effect of central administration of cannabinoid antagonist, SR141617A on feeding behavior, and on the ability of capsaicin-induced deafferentation to prevent changes in feeding elicited by peripheral administration of cannabinoid drugs [12]. The involve-

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ment of peripheral endocannabinoids in the control of feeding has been supported by observation, that anandamide is synthesized within gut tissues, with intestinal concentration increasing in 24 hours fasted rats [12]. These findings suggest that stimulation or blockade of peripheral CB1 receptors may influence central motivational processes, and have been interpreted as indicating a possible role for peripheral anandamide as "hunger signal".

Several data suggest a relationship between the cannabinoid system and the gastrointestinal peptides involved in food intake and apatite regulation [12,15]. Cummings *et al.* [25] reported an increase of plasma ghrelin concentration during fasting and rapid decrease during meal consumption in humans. Recently Cani et al [15] have shown that an increase of ghrelin in fasted rats was abolished by selective CB1 receptors antagonist, SR161417A. This is in agreement with our observations point to an increase of plasma ghrelin level after a single ip injection of R-(+)-methanandamide and CP 55,940. The localization of CB1 receptors in the mucosal tissue of somach fundus [15], and ghrelin synthesis occurring in this part of stomach [18] are in favor of a physiological interaction between these two systems. Also Konturek *et al.* [36] have been recently suggested the interaction between cannabinoid and ghrelin signaling pathways in the regulation of food intake by the brain-gut axis.

It is noteworthy that gastric and intestinal vagal afferents that express CB1 receptors also express receptors for the anorexigenic peptide cholecystokinin (CCK) [11]. Expression of vagal CB1 receptors is increased by fasting and is diminished by reffeding. Moreover, CCK, which is released by food and believed to act as a satiety signal, also decreases CB1 receptors expression in vagal affrent neurons [11]. Thus, it is possible that appetite may be modulated by interactions between the peptide and cannabinoid signals originating in the periphery. It has been reported that CB1 receptor agonist, WIN 55,212-2 inhibited potassium-evoked CCK release from hippocampal slides [37]. Moreover, recently the suppression of plasma ghrelin concentration after intravenous CCK-8 infusion was observed [38]. Since CB1 receptor agonists inhibit CCK release, and CCK suppresses ghrelin secretion, it seems to be possible that observed in the present study the enhancement of ghrelin secretion after cannabinoids injection was evoked by the inhibition of CCK suppression on ghrelin release (disinhibitory effect). This possibility is in accordance with the observation based on immunhistochemical study performed by Burdyga *et al.* [19], directed at GHS-R1a of the ghrelin receptors, that revealed the expression of these receptors in over 75% of neurons in nodose ganglia expressing CCK-1 receptors. The latter authors suggest that the actions of CCK and ghrelin are mediated by a common population of vagal afferent neurons.

Although, there is an increasing evidence for a peripheral role of the cannabinoid system in food-consumption behavior, the molecular mechanism of different cannabinoid-ralated molecules action on food intake through their influence on gastrointestinal tract remains to be fully elucidated.

The present study suggests that the modulation of the secretion of gastrointestinal orexigenic peptides such as ghrelin, by the peripheral cannabinoid system, mainly expressed in the upper part of the gastrointestinal tract, may participate in food regulation.

In conclusion, our study indicates that stimulation of appetite exerted by cannabinoids might be connected with an increase of ghrelin secretion from gastric X/A-like cells.

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