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Exogenous obestatin affects pancreatic enzyme secretion in rat through two opposite mechanisms, direct inhibition and vagally-mediated stimulation

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ABSTRACT. It is suggested that obestatin can stimulate the secretion of pancreatic juice in rats and this effect is abolished by vagotomy. Thus, the aim of the present study was to further elucidate the mechanism by which obestatin controls the exocrine pancreas secretion. Anesthetized male Wistar rats (200 ± 15 g body weight) were administered intravenously (iv) and intraduodenaly (id) every 30 min obestatin in boluses of 30, 100 and 300 nmol · kg^{-1} body weight and 15 min later pancreatic-biliary juice (PBJ) was collected to determine the PBJ volume, total protein and enzymes activity. Obestatin injections were also done following subdiaphragmatic vagotomy, capsaicin deafferentation and pharmacological blockage of the mucosal cholecystokinin 1 (CCK1) receptor with tarazepide. Dispersed acinar cells were isolated from rat pancreas by collagenase digestion, stimulated with CCK-8 (10^{-10} M) and incubated with obestatin (10^{-9}–10^{-6} M) *in vitro*. It was noted that iv and id obestatin administrations did not affect the PBJ volume but increased protein and trypsin outputs regardless way of administration, and amylase and lipase outputs after id injection. Similarly to vagotomy, the capsaicin and tarazepide pre-treatments abolished the effects of obestatin. In contrast to the *in vivo* experiment, the treatment of dispersed pancreatic cells with the CCK-8+obestatin combination showed that obestatin decreased the CCK-8-stimulated amylase release from acinar cells *in vitro*, but obestatin alone did not exert effect on amylase release. So, it is thought that obestatin can stimulate the exocrine pancreas secretion *via* an indirect vagal mechanism, whilst its direct action on the acinar cells is also possible but with the opposite effects.

Introduction

Pancreas is a gland consisting of cells with two profiles of excretion: exocrine (digestive enzyme secretion from acinar cells) and endocrine (hormone release from islets). The pancreas is also built of a dense network of capillaries, which allows a quick release of hormones into the bloodstream. Whereas the digestive enzymes are secreted directly into the duodenum lumen. The pancreas is innervated

by sympathetic and parasympathetic nerves (Chandra and Liddle, 2009). The parasympathetic efferent fibres originate from the dorsal motor nucleus of the vagus nerve (DMV) localized in the brain stem. Pancreatic secretion is regulated by several neurotransmitters, and different regulatory peptides and hormones (Chandra and Liddle, 2009). The main hormone that regulates exocrine pancreatic secretion is cholecystokinin (CCK) that can bind to the CCK1 receptor localized on pancreatic acinar cells and stimulate the release of digestive enzymes. CCK can also bind to the CCK1 receptor on capsaicin-sensitive C-type vagal afferent nerves and generate a signal sent to the brain stem from where it is transmitted to the pancreas (Wright et al., 2011; Campos et al., 2012; Bülbül et al., 2017). As found previously, the group of intestinal regulatory peptides that affect digestive function includes, among others, ghrelin, leptin, apelin and obestatin (Matyjek et al., 2004; Kapica et al., 2008; Antushevich et al., 2016).

For the first time, obestatin was isolated from rat stomachs in 2005 (Zhang et al., 2005). This widely expressed in the body a 23-amino acid peptide is encoded on preproghrelin gene region. Obestatin has been found in the small intestine, colon, spleen, cerebral cortex, skeletal muscle, adipose tissue, lung, liver and many other tissues, but the major production of obestatin in rats takes place in the fundic region of gastric mucosa (Zhang et al., 2005; Zhao et al., 2008; Grönberg et al., 2010; Moretti et al., 2014). In the pancreas, obestatin-immunoreactive cells are mainly present in the peripheral region of the islets and do not co-localize with glucagon, pancreatic polypeptide Y or insulin secreting cells (Zhao et al., 2008). Fasting and gastric bypass surgery do not affect the obestatin-producing cells in the stomach, but fundectomy and gastrectomy drastically diminish its plasma level (Matyjek et al., 2004; Furnes et al., 2008).

In contrast to obestatin peptide, obestatin receptor is still unknown, nevertheless, a series of experiments performed on rats and mice provided scientific evidence for obestatin action in the organism. Obestatin was shown to increase memory retention and inhibit drinking behaviour (Samson et al., 2007). In conscious rats, intravenous (iv) injections of obestatin inhibited antral and duodenal motor activity through a vagal afferent-dependent pathway (Ataka et al., 2008). The effect of obestatin on the endocrine pancreas activity is differently described in the literature. In the study of Green et al. (2007) on mice no effect of obestatin on insulin secretion was found, whilst Egido et al. (2009) using perfused rat pancreas demonstrated either stimulation or inhibition of insulin release with 1 and 10 nM of obestatin, respectively. In turn, Qader et al. (2008) reported that secretion of insulin is inhibited by obestatin in a concentration-dependent manner in both mouse and rat islets, while the secretion of glucagon can be stimulated. Moreover, obestatin impeded the secretion of both somatostatin and pancreatic polypeptide in the manner similar to acyl ghrelin (Qader et al., 2008). In several studies it was shown that obestatin exhibits some protective and therapeutic effects in the gut (Ceranowicz et al., 2015; Bukowczan et al., 2016). Preventive administration of obestatin inhibited the development of cerulein- and ischemia/reperfusion-induced acute pancreatitis (Ceranowicz et al., 2015). Moreover, it was observed that obestatin promoted survival of pancreatic islets, in particular β-cells (Granata et al., 2008).

In our previous study it was shown that in contrast to ghrelin (Kapica et al., 2006), exogenous obestatin can stimulate the secretion of pancreatic enzymes, and this effect can be abolished by vagotomy (Kapica et al., 2007). Therefore, the aim of the present study was to further elucidate the mechanisms by which exogenous obestatin can control the secretion of pancreatic enzymes using *in vivo* and *in vitro* rat models.

Material and methods

Animals

The experiments were approved by the II Local Ethics Committee for the Experiments on Animals in Lublin (Poland), agreement No. 32/2006. In total, 74 Wistar male rats weighing 200 ± 15 g were used in the *in vivo* and *in vitro* experiments. The animals were housed in a light- and temperature-controlled room with free access to standard feed (Agropol, Motycz, Poland) and water. The rats were housed in plastic cages (2 rats per cage) with sawdust bedding.

Animal preparation for *in vivo* study

A night before *in vivo* experiment the animals were fasted. The surgery was performed in 60 rats under mixed xylazine (12 mg · kg^{-1} body weight (BW); Rometar, Bioveta a.s., Ivanovice na Hané, Czech Republic) and ketamine (35 mg · kg^{-1} BW; Bioketan, Vetoquinol Biowet Sp. z o.o., Gorzów Wielkopolski, Poland) intramuscular anaesthesia. Body temperature was continuously controlled and maintained by heating lamps. The right external jugular vein was prepared, and silicone tubing was inserted and fixed with ligatures. Continuous intravenous (iv) infusion of saline (0.9% NaCl, peristaltic pump speed

2 ml · h^{-1}) started immediately after cannulation and continued until the end of the experiment. The infusion was stopped during the iv obestatin injections. Following midline laparotomy, polyethylene tubing was inserted into the common pancreatic-biliary duct for collection of bile and pancreatic juice mixture (PBJ). The second polyethylene tubing was inserted into the duodenum through the pylorus for the reintroduction of PBJ and obestatin administration. The PBJ was reinstilled into the duodenum at the rate of its secretion through the cannula, and obestatin boluses were administered as described below. The anaesthesia was maintained during the experiment by intraperitoneal (ip) administration of anaesthetics. The involvement of vagal nerves was examined following subdiaphragmatic vagotomy and capsaicin deafferentation as described by Kapica et al. (2006), Ryu et al. (2010) and Peters et al. (2013). The involvement of the duodenal mucosal CCK1 receptor was studied (Zabielski et al., 1998) following intraduodenal (id) administration of a selective CCK1 receptor antagonist – tarazepide (generously supplied by Solvay Pharmaceuticals GmbH, Hanover, Germany).

In vivo experimental protocol

Collection of PBJ started immediately after the surgery. PBJ was collected at 15 min intervals into 1.5 ml polyethylene tubes held on ice. Vehicle (0.9% NaCl) and three obestatin boluses (30, 100 and 300 nmol · kg^{-1} BW, synthetic obestatin, Yanaihara Institute Inc., Fujinomiya-shi, Japan) were administered iv or id every 30 min. For each way of obestatin administration 5 series of experiments (6 rats in each) were performed to test the effect of obestatin on PBJ secretion: control (animals after sham operation and iv or id 0.9% NaCl infusion), obestatin (animals after sham operation and iv or id obestatin boluses infusion), capsaicin (animals with capsaicin pre-treatment and obestatin boluses infusion), tarazepide (animals with tarazepide pretreatment and obestatin boluses infusion), vagotomy (animals after vagotomy and obestatin infusion).

For each rat the PBJ samples collected during 15-min intervals after 0.9% NaCl (obestatin 0) and then after each obestatin dose injections (obestatin 30, obestatin 100 and obestatin 300) were used for further analysis. The PBJ samples were checked for their weight and 0.1 ml samples were stored at −20 °C. The samples were analysed for total protein using the Lowry method performed on 96-well microwell plates with bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as the standard. The intra- and inter-assay variations for the protein determination were 3.1 and 3.6%, respectively. Trypsin (EC 3.4.21.4) activity was estimated after enterokinase (Sigma-Aldrich, St. Louis, MO, USA) estimation using N-alpha-benzoyl-DL-arginine-*p*-nitroanilide (Sigma-Aldrich, St. Louis, MO, USA) as the substrate (Kapica etal., 2006). The intra- and inter-assay variations for the trypsin activity determination were 2.8 and 3.2%, respectively. Amylase (EC 3.2.1.1) activity was measured according to Bernfeld (1955) with minor modification by Jankowska et al. (2007). Lipase (EC 3.1.1.3) activity was determined according to the method described by Furukawa et al. (1982) with absorbance of samples read at a wave length of 412 nm after 10 and 20 min (QuantiChrom™ Lipase Assay Kit, Cat. No. DLPS-100; BioAssay Systems, Hayward, CA, USA).

Preparation of dispersed acinar cells and *in vitro* study protocol

Dispersed acinar cells were obtained from rat pancreas by collagenase digestion according to the method proposed by Jankowska et al. (2007). In total 14 male Wistar rats were euthanatized by CO_2 inhalation. The pancreatic tissue was immediately isolated and placed in ice cold 0.9% NaCl. Small pieces of pancreas were transferred to fresh Ringer-Krebs-HEPES buffer (RKH) containing: 5 mM glucose, 25.2 mM HEPES, 1.5 mM glutamine, 1.5 mg · ml^{-1} bovine serum albumin (BSA), 0.1 mg · ml^{-1} soyabean trypsin inhibitor, 103 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.1 mM $MgSO_4$, 1% (v/v) Eagle's Minimum Essential Medium. Basal Medium Eagle was oxygen-saturated and adjusted to pH 7.4. All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Next, the pancreatic tissue was digested twice in the RKH buffer supplemented with 100 U · ml^{-1} collagenase (Worthington Biochem. Corp., Lakewood, NJ, USA) for 15 and 20 min. After that, the tissue was passed through a 170 µm cell dissociation steel sieve. The cells were purified by suspension in RKH and centrifugation at 40 *g*, 4 °C for 30 s (3 times) and by sedimentation in 4% BSA in RKH. The cell suspension was examined by light microscopy and approximately 10^5 cells per 1 ml were added to vials containing experimental factors: 10^{-10} M of CCK-8 (CCK-octapeptide, 26–33 sulphated form; Sigma-Aldrich, St. Louis, MO, USA) and from 10^{-9} to 10^{-6} M of obestatin (Yanaihara Institute Inc., Fujinomiya-shi, Japan). The following groups were obtained: control (without obestatin/CCK-8), obestatin-10^{-9} M, obestatin-10^{-8} M, obestatin-10^{-7} M, obestatin-10^{-6} M, CCK-8 (without obestatin), CCK-8+obestatin-10^{-9} M, CCK-8+obestatin-10^{-8} M, CCK-8+obestatin-10^{-7} M, CCK-8+obestatin-10^{-6} M.

The vials were incubated under O_2 saturation at 37 °C in a shaking water bath for 40 min. The reaction was stopped by placing the vials on ice and separating the cells by centrifugation at 100 *g*, 4 °C for 5 min. Amylase release from the acinar cells was evaluated by measuring the amylase activity in the supernatant according to method described by Bernfeld (1955). Total protein was determined with the Lowry method. Amylase release was expressed as amylase activity in supernatant per mg of total protein in cellular sediment in each vial. The result for each group was then calculated in relation to control group (without obestatin/CCK-8) which was regarded as 0. Protein content and amylase activity were analysed in 3 or 8 replicates.

Statistical analysis

The results of the *in vivo* study were calculated as means (± SEM) for the 15 min PBJ collection intervals. The data pertaining to the effect of obestatin on PBJ were expressed in absolute values. One-way analysis of variance (ANOVA) for repeated measures was followed by Tukey's post-hoc test and linear trend analysis (GraphPad Prism v.3, Graph Pad Software, San Diego, CA, USA) to investigate the relationships between the dose of obestatin and PBJ secretory response. For statistical analysis of the results obtained from the *in vitro* part of the study two tests were used. To indicate the differences between each examined group and control group the Student t-test was performed. To compare all examined groups (without control one) one-way ANOVA with Tuckey's post-hoc test was used. A value of $P < 0.05$ was considered statistically significant.

Results

In order to show possible mechanisms of obestatin action on the pancreatic secretion, it is necessary to present the obestatin effect in non-operated animals and in animals after vagotomy. These results have been already published by Kapica et al. (2007), however to obtain the whole coherent picture of mechanisms some results are repeated (the repetitions are clearly underlined).

As it was already presented the iv infusion of obestatin increased protein and trypsin outputs in a dose-dependent manner (test for linear trend $P < 0.0001$) but did not affect the PBJ volume (taken from Kapica et al. (2007), Figure 1A–C, series II). The protein response to 30–300 nmol · kg^{-1} BW obestatin constituted to 35–60% of that observed following the iv infusion of 12 pmol · kg^{-1} BW CCK-8 (data not shown). The vagotomy abolished pancreatic

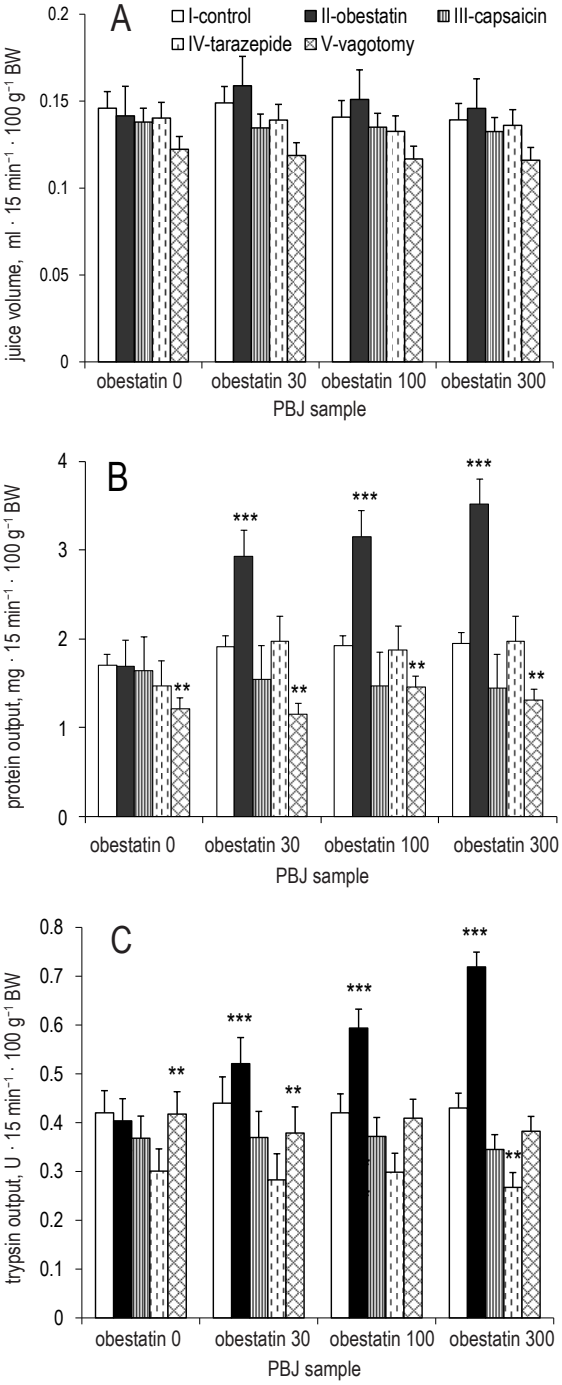

Figure 1. Effect of intravenous (iv) bolus infusions of obestatin (0, 30, 100 and 300 nmol · kg^{-1} body weight, BW) administrated every 30 min on pancreatico-biliary juice (PBJ) volume (A), protein output (B) and trypsin output (C) in anaesthetized rats

For each time point (obestatin 0, obestatin 30, obestatin 100 and obestatin 300), there are presented 5 series: I – control (animals after sham operation and 0.9% NaCl infusion), II – obestatin (animals after sham operation and obestatin infusion), III – capsaicin (animals with capsaicin pre-treatment and obestatin infusion), IV – tarazepide (animals with tarazepide pre-treatment and obestatin infusion), V – vagotomy (animals after vagotomy and obestatin infusion). Each bar represents a 15-min PBJ sample after iv infusion of 0.9% NaCl or obestatin. Data are presented as mean ± SEM (n = 6). The * indicates that the value is significantly different from respective control in each time point (one-way ANOVA for repeated measurements followed by Tukey's test: * – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$)

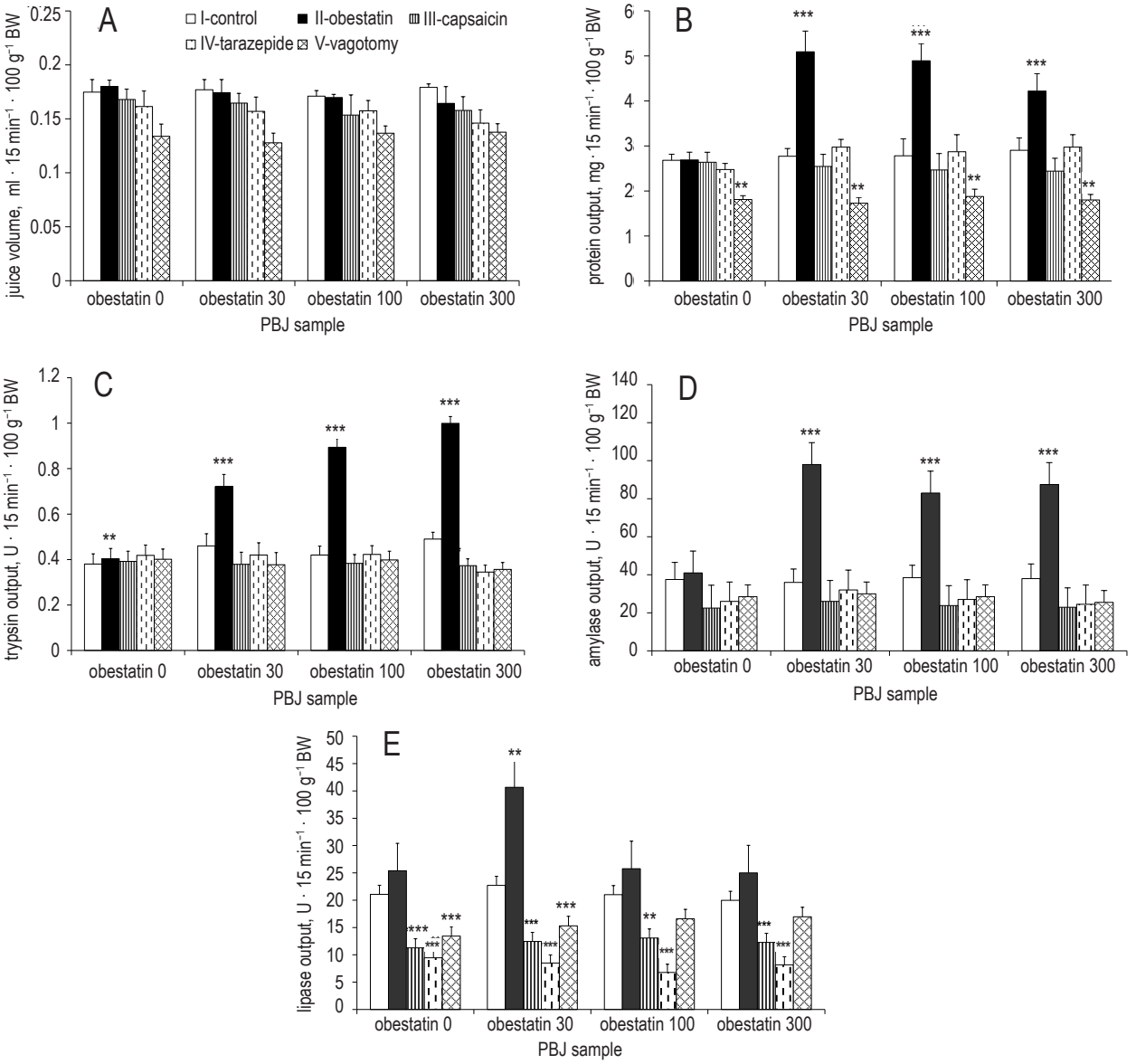

Figure 2. Effect of intraduodenal (id) bolus infusions of obestatin (0, 30, 100 and 300 nmol · kg^{-1} body weight, BW) administrated every 30 min on pancreatico-biliary juice (PBJ) volume (A), and protein (B), trypsin (C), amylase (D) and lipase (E) output in anaesthetized rats.
For each time point (obestatin 0, obestatin 30, obestatin 100 and obestatin 300), there are presented 5 series: I – control (animals after sham operation and 0.9% NaCl infusion), II – obestatin (animals after sham operation and obestatin infusion), III – capsaicin (animals with capsaicin pre-treatment and obestatin infusion), IV – tarazepide (animals with tarazepide pre-treatment and obestatin infusion), V – vagotomy (animals after vagotomy and obestatin infusion). Each bar represents a 15-min PBJ sample after id infusion of 0.9% NaCl or obestatin. Data are presented as mean ± SEM (n = 6). The * indicates that the value is significantly different from respective control in each time point (one-way ANOVA for repeated measurements followed by Tukey's test: * – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$)

responses (protein and trypsin output) to iv obestatin (results also already presented in Kapica et al. (2007), Figure 1A–C, series V). The deafferentation with capsaicin and blockage of the mucosal CCK1 receptor also totally abolished pancreatic responses to iv obestatin (Figure 1A–C, series III and IV).

As it was presented by Kapica et al. (2007), also the id infusion of obestatin increased protein and trypsin outputs but did not affect the PBJ volume (taken from Kapica et al. (2007), Figure 2A–C, series II). Now, it was shown that the id infusion of obestatin increased also amylase and lipase outputs (Figure 2D and 2E, series II). The effect on the trypsin output of the studied pancreatic enzymes was dose-related (test for linear trend $P < 0.001$). There was no significant difference between the protein outputs in response to the obestatin doses applied in the present study. The responses for the first two obestatin doses were stronger than the similar doses given iv (Kapica et al., 2007). Similar to iv infusions, the subdiaphragmatic vagotomy abolished the id obestatin effect on protein and trypsin output (taken from Kapica et al., 2007; Figure 2B and 2C, series V) but also on amylase and lipase output (Figure 2D

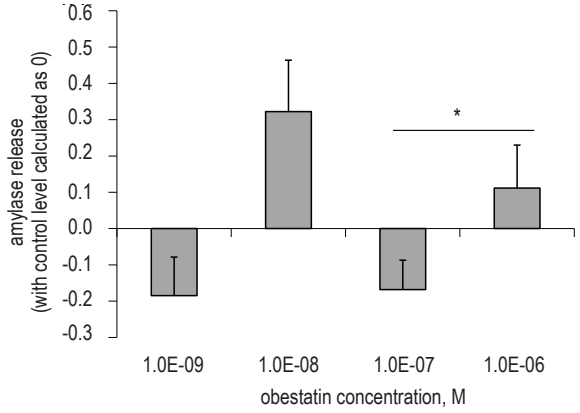

Figure 3. The effect of obestatin on amylase release from rat dispersed pancreatic acinar cells *in vitro*

The amylase release was calculated as amylase activity in supernatant per mg of total protein in cellular sediment in each vial. The result for each group was then calculated in relation to control group (without obestatin/CCK-8) which was regarded as 0 (the control bar is not presented on the figure). The results are expressed as mean ± SEM taken from 3 separate experiments (n = 9 for all 3 experiments). The * above the line indicates that the groups are statically different according to ANOVA with Tuckey's post-hoc test (* – $P < 0.05$)

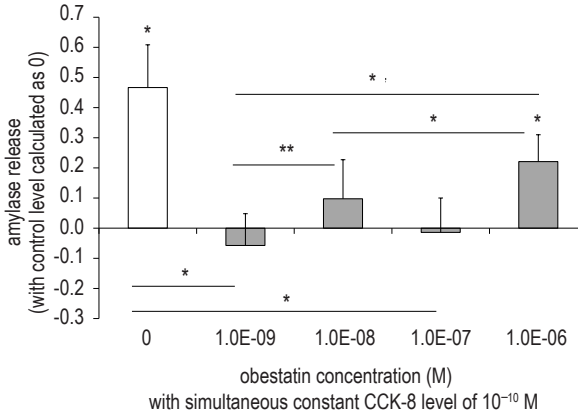

Figure 4. The effect of obestatin and CCK-8 on amylase release from rat dispersed pancreatic acinar cells *in vitro*

The amylase release was calculated as amylase activity in supernatant per mg of total protein in cellular sediment in each vial. The result for each group was then calculated in relation to control group (without obestatin/CCK-8) which was regarded as 0 (the control bar is not presented on the figure). The results are expressed as mean ± SEM taken from 3 separate experiments (n = 9 for all 3 experiments). The * above the line indicates that the groups are significantly different according to ANOVA with Tuckey's post-hoc test. The * above the bar indicates that the group is significantly different from control group according to Student t-test (* – $P < 0.05$; ** – $P < 0.01$)

and 2E, series V). The deafferentation with capsaicin also block the obestatin stimulated effect on all examined parameters to the control level of protein, trypsin and amylase (Figure 2B–2D, series III) and below this level for lipase (Figure 2E, series III). Also the same effect was observed in case of blockage of the mucosal CCK1 receptor (Figure 2B–2E, series IV).

The results obtained in *in vitro* study showed that obestatin alone did not stimulated amylase release from dispersed pancreatic cells; however the difference was observed between obestatin-10^{-6} M and obestatin-10^{-7} M groups (Figure 3). The CCK-8 treatment alone stimulated amylase release in comparison with control group (Figure 4). The simultaneous obestatin addition inhibited CCK-8-stimulated amylase release in groups CCK-8+obestatin-10^{-9} M and CCK-8+obestatin-10^{-7} M to control level. The amylase release in CCK-8+obestatin-10^{-6} M group was higher than in control, CCK-8+obestatin-10^{-9} M and CCK-8+obestatin-10^{-8} M groups. The difference in amylase release was also stated between CCK-8 +obestatin-10^{-9} M and CCK-8+obestatin-10^{-8} M.

Discussion

Previously, it was shown that exogenous obestatin can stimulate the secretion of pancreatic protein and trypsin outputs in anesthetized rat. The stimulation was observed in response to both iv and id administration of obestatin and was abolished by vagotomy (Kapica et al., 2007). Obestatin did not affect the secretion of water showing a similar pattern to that usually observed after administration of cholecystokinin. The results of the present study clearly indicate that the stimulation by exogenous obestatin observed *in vivo* is not the result of a direct action on the pancreatic acini. In our *in vitro* pancreatic acinar cell preparation, obestatin reduced the release of CCK-8-stimulated amylase. This suggests that possible obestatin receptor must be present on pancreatic acinar cells, like the ghrelin receptor. However, the iv administration of obestatin dose-dependently stimulated the secretion of pancreatic enzymes (trypsin, lipase and amylase) *in vivo*. This, in turn, indicates that the direct effect of obestatin on acinar cells *in vivo* is of much lesser importance for controlling pancreatic exocrine secretion, and the secretion is actually stimulated by some indirect mechanism that needs entirely functional vagal nerves. In this study the obestatin doses much smaller than those used by Zhang et al. (2005) were enough to obtain similar effects. The PBJ protein output in response to the highest dose of iv obestatin was fixed within a range usually observed in our laboratory during the continuous iv infusions of CCK-8 in a dose of 12 pmol · kg^{-1} BW per h (Matyjek et al., 2004; Kapica et al., 2006). This dose has been considered to provide physiological stimulation of pancreatic secretion in rats (Li and Owyang, 1996). Moreover, all responses to iv administration of obestatin were abolished following the pre-treatment with the selective CCK1 receptor

antagonist given in a dose that blocks the mucosal CCK1 receptor in the duodenum and upper jejunum (Zabielski et al., 1998). The responses were also abolished following the deafferentation with capsaicin and subdiaphragmatic vagotomy, suggesting that obestatin acts on the exocrine pancreas through the duodenal CCK1-vagal mechanism (Zabielski et al., 1998; Konturek et al., 2003). It has already been documented that some regulatory peptides, such as leptin, ghrelin and apelin, can control pancreatic secretion by influencing this neurohormonal mechanism (Matyjek et al., 2004; Kapica et al., 2006, 2008).

To our knowledge, the release of obestatin into the lumen of the gastrointestinal tract has not been evidenced yet. On the other hand, the releases of the sister peptide ghrelin (Date et al., 2000), leptin (Sobhani et al., 2000), gastrin, CCK and many others (Rao, 1991) have been already shown. The release of obestatin from gastric mucosa A-type cells into the lumen is quite possible. In our study it was shown that luminal obestatin can stimulate pancreatic secretion through the neurohormonal mechanism, which associates the mucosal CCK1 receptor and long vago-vagal reflex (Konturek et al., 2003) like that infused systemically. Nevertheless, further studies are needed to better understand the biological role of luminally released regulatory peptides in maintaining gastrointestinal homeostasis, cytoprotection and regulation of digestive processes. One of possible roles of obestatin is to maintain pancreatic enzyme secretion during the interdigestive and starvation periods in alliance with other gut regulatory peptides and vagal activity. For instance, in rats starved for 20 h id infusion of orexin-B increased the secretion of pancreatic juice volume (Korczynski et al., 2006). Interruption of vagal activity is known to reduce the interdigestive secretion of pancreatic juice, while stimulation thereof enhances the secretion.

Obestatin is considered to exhibit opposite biological functions to ghrelin (Zhang et al., 2005); however the results of the studies on exocrine activity of pancreas in rats showed that sometimes obestatin and ghrelin effect can be the same. Indeed, iv infusions *in vivo* led to achieve opposite results – inhibition of enzyme protein secretion by pentaghrelin (Kapica et al., 2006) and stimulation by obestatin (present study). In contrast, the direct action on the rat pancreatic acinar cells of both pentaghrelin (Jankowska et al., 2007) and obestatin (present study) led to the reduction of CCK-8-stimulated amylase release. Additionally, id administration of ghrelin (Nawrot-Porąbka et al., 2007) and obestatin (present study) resulted in the stimulation of the exocrine pancreas activity.

Conclusions

Obestatin can stimulate the exocrine pancreas secretion *via* an indirect vagal mechanism regardless hormone way of administration (intravenous or intraduodenal), whilst the direct action of obestatin on pancreatic acinar cells is also possible but with opposite – inhibiting – effect.

Acknowledgments

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