The effect of pancreatic polypeptide on gastric accommodation and gastric emptying in conscious rats

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Running head: Pancreatic polypeptide and gastric motility in rats

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Abstract

Introduction. Pancreatic polypeptide (PP) is an anorexigenic hormone released from pancreatic F-cells upon food intake. We aimed to determine the effect of PP on gastric accommodation and gastric emptying in conscious Wistar HAN rats to investigate whether effects on motor function could contribute to its anorexigenic effects. Methods. Intragastric pressure (IGP) was measured through a chronically implanted gastric fistula during the infusion of a nutrient meal (Nutridrink®; 0.5 ml min⁻¹). Rats were treated with PP (0, 33 and 100 pmol kg⁻¹ min⁻¹) in combination with L-NG-arginine methyl ester (L-NAME; 180 mg kg⁻¹ h⁻¹), atropine (3 mg kg⁻¹ h⁻¹) or vehicle. Furthermore, the effect of PP was tested after subdiaphragmal vagotomy of the stomach. Gastric emptying of a non-caloric and a caloric meal after treatment with 100 pmol kg⁻¹ min⁻¹ PP or vehicle was compared using X-rays. Results. PP significantly increased IGP during nutrient infusion compared to vehicle (p<0.01). L-NAME and atropine significantly increased IGP during nutrient infusion as compared to vehicle treatment (p<0.005 and <0.01 respectively). The effect of PP on IGP during nutrient infusion was abolished in the presence of L-NAME and in the presence of atropine. In vagotomized rats, PP increased IGP compared to intact controls (p<0.05). PP significantly delayed gastric emptying of both a non-caloric (p<0.05) and a caloric meal (p<0.005). Conclusion. PP inhibits gastric accommodation and delays gastric emptying, probably through inhibition of nitric oxide release. These results indicate that, besides the well-known centrally-mediated effects, PP might decrease food intake through peripheral mechanisms.

Keywords

Gastric motility, pancreatic polypeptide, gastric emptying
Introduction

Between meals, gastric smooth muscle maintains a high resting tone, due to the myoelectrical properties of the smooth muscle but also because of a constant cholinergic input from the vagal nerves. The stomach is known to relax upon food intake. This reflex relaxation, also referred to as gastric accommodation (GA), enables the stomach to receive large quantities of food without an increase in intragastric pressure (IGP) [35]. Relaxation of the stomach upon food intake is mediated via vago-vagal reflex pathways, eventually leading to activation of mainly nitrergic nerves in the enteric nervous system [38]. Nitric oxide (NO) finally relaxes the smooth muscle cells and the gastric tone decreases [19].

We recently described a method to assess GA in rats by measuring the IGP during intragastric infusion of a liquid test meal [15]. During infusion of a test meal, IGP increased initially until an inflection point was reached, after which the IGP stabilized despite further gastric distension. It was suggested that this inflection point represents the onset of GA, which is represented by the plateau phase. Pretreatment with N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME), a NO synthase (NOS) inhibitor, resulted in a higher increase of IGP, confirming the role of NO as the main mediator of GA.

Gastric function is regulated by a variety of gastrointestinal hormones. Pancreatic polypeptide (PP), a member of the PP family, is a 36 amino acid hormone produced by F-cells within the pancreatic islets. It is released upon food intake or in response to other gastrointestinal peptides and its secretion is mediated through a vagal cholinergic mechanism, as the PP response to a meal or cholecystokinin (CCK) infusion is reduced after treatment with atropine or after truncal vagotomy [27, 33]. Alterations in postprandial PP release have been demonstrated in clinical syndromes associated with abnormal eating behavior, such as Prader-Willi syndrome (decreased PP secretion), anorexia nervosa (increased PP secretion) and obesity (decreased PP secretion) [11, 23, 40]. Exogenous PP has been shown to reduce food intake in humans (both healthy volunteers and Prader-Willi subjects), dogs and mice [5, 6, 24, 34]. These observations indicate that PP is involved in the regulation of food intake.
In rats, it has been shown that intravenous (i.v.) infusion of PP caused an increase in antral motility as measured with strain gauges [26]. This increase was abolished by pretreatment with atropine and when the animals were vagotomized, suggesting that PP acts through a vagal cholinergic mechanism. The same outcome was seen after micro-injection of PP into the dorsal vagal complex (DVC), again supporting the theory that PP can affect antral motility through vagal pathways [25].

In humans, gastric emptying (GE) was delayed after PP treatment [32]. Also in mice receiving PP and in PP-overexpressing mice a decreased GE rate was observed [3, 20]. The gold standard to assess GE in animals makes use of inert markers that in some cases are mixed with a meal and for which the animal has to be sacrificed to determine the gastric emptying rate. A disadvantage of this technique is that no paired measurements can be performed.

As PP is considered to be involved in gastric motility and food intake, we aimed to study the effect of PP on GA during nutrient infusion in conscious rats. To investigate the pathway through which PP exerts its effect, we tested the influence of L-NAME, the muscarinic receptor antagonist atropine and we performed selective vagotomy of the stomach. In a second series of experiments we aimed to evaluate GE after PP treatment using X-rays to visualize the meal. The results from this study might help us further understand how PP affects food intake and satiety.
Methods and materials

Animals

23 Male Wistar HAN IGS rats (Charles River, L’Abresle Cedex, France) were used for this study. Animals were single-housed in polypropylene cages in a temperature- and humidity controlled environment, with a light/dark cycle of 12h and free access to water and food pellets. At the start of the experiments rats weighed 200-250g. To reduce restraint stress and motion artefacts, rats were regularly accustomed to custom-made Bollmann cages for 30 minutes a day during the week prior to the start of the experiments. Experiments were approved by the ethical committee for animal use of the faculty of Medicine of the University of Leuven.

a) Gastric accommodation study

Surgery

Fistula implantation. Gastric fistulas were chronically implanted in all animals at least 2 weeks before the start of the experiments, as previously described by Janssen et al. [15]. In short, rats were anaesthetized using inhalation anaesthesia (2.5 % Isoflurane). A custom-made fistula (made of acryl plastic; outer diameter of 5.5 mm, inner diameter 4.0 mm and a length of 16.0 mm) was inserted into the stomach through a midline incision of the abdomen. The fistula was attached along the major curvature of the stomach and further exteriorized through the abdominal muscle and the skin. The midline incision was closed and the skin was sutured. To suppress pain, animals recieved 0.8 ml kg⁻¹ of buprenorphin (Temgesic®, Reckitt Benckiser Healthcare, Hull, UK; 0.3 mg ml⁻¹) during surgery. Also an injection of an electrolyte solution (Lactetrol®, Eurovet, Heusden-zolder, Belgium) was given to aid recovery after the surgery. From 3 days prior to surgery until 4 days after the surgery, enrofloxacin (Baytril® 10%, Bayer, Diegem, Belgium) was added to the drinking water (2 ml liter⁻¹). For at least 2 weeks rats were left to recover from the operation while closely monitoring their health status.

Vagotomy. In a subset of rats sub-diaphragmatic denervation was performed prior to fistula implantation during the same surgical procedure. After opening of the
abdominal cavity the esophagus was isolated. Nerve bundles running along the esophagus towards the stomach were removed over a length of 3mm using microsurgical instruments under an operating microscope (magnification 25x).

Experimental protocol

The experimental protocol was similar to that used in Janssen et al. [15]. Animals were fasted overnight before the beginning of each experiment to prevent the presence of food particles in the stomach, access to water was provided ad libitum. At the start of the experiment the rats were weighed and then shortly anesthetized to open the fistula and to remove any remaining gastric contents with a syringe. An i.v. line in the tail vein was prepared for the infusion of the test drugs. The opened fistula was connected to a meal infusion system containing a syringe pump to determine the infusion speed. The meal, a liquid nutrient (Nutridrink®, 1.5 kcal ml⁻¹; Nutricia, Bornem, Belgium), was pre-warmed to 37 °C before infusion into the stomach. A water-filled polyethylene catheter (inner diameter: 0.86 mm, outer diameter: 1.27 mm), connected to an external pressure transducer (Siemens Elema 746, Siemens, Iselin, New Jersey, USA), was positioned 3-5 mm into the stomach through the fistula. Customized computer software (WinDaq Lite Data Acq DI-7x0 USB0) was used to record IGP.

After connection of the fistula rats were positioned in a Bollmann cage and allowed to wake up. After a stabilization period of 15 minutes after the rats regained consciousness, i.v. infusion of a treatment started. 15 minutes thereafter, the liquid nutrient meal was infused into the stomach at a rate of 0.5 ml min⁻¹ for 20 minutes. Five minutes after the end of the nutrient infusion, all tubes were disconnected from the fistula, the fistula was closed, and the rats were returned to their cages.

Drugs & treatments

The following test drugs were used: PP (Polypeptide Group, Strasbourg, France), L-NAME (Sigma-Aldrich, Bornem, Belgium), and atropine sulphate salt monohydrate
PP, L-NAME and atropine sulphate salt monohydrate were dissolved in 0.9% saline. The treatments used were PP (33 and 100 pmol kg\(^{-1}\) min\(^{-1}\)), L-NAME (180 mg kg\(^{-1}\) h\(^{-1}\)), PP (100 pmol kg\(^{-1}\) min\(^{-1}\)) + L-NAME (180 mg kg\(^{-1}\) h\(^{-1}\)), atropine (3 mg kg\(^{-1}\) h\(^{-1}\)), PP (100 pmol kg\(^{-1}\) min\(^{-1}\)) + atropine (3 mg kg\(^{-1}\) h\(^{-1}\)). As vehicle treatment saline 0.9% was used. All drugs were infused intravenously via the tail vein. In vagotomized rats only vehicle and the high dose of PP were tested.

IGP analysis

IGP was represented as an increase from baseline, which was the mean IGP during the 5 minutes preceding nutrient infusion start. To filter out movement artefacts, the data were transformed by calculating the moving median over one minute.

b) Gastric emptying study

Experimental protocol

After an overnight fast with \textit{ad libitum} access to water, 6 rats were shortly anesthetized to prepare an iv line in the tail vein for the infusion of vehicle or PP (100 pmol kg\(^{-1}\) min\(^{-1}\)). 15 min after the start of the infusion, rats were gavaged with 3 ml of a caloric meal (Nutridrink\textsuperscript{®}) or 3 ml of a non-caloric meal (tap water). Hydroxypropylmethylcellulose (0.01 g ml\(^{-1}\)) was added to the water to match viscosity of Nutridrink (20 mPa s). BaSO\(_4\) (0.5 g ml\(^{-1}\)) was added to both meals as a radiocontrast. Animals were placed in a custom-made Bollmann cage and placed onto the platform of a mammographic device (Embrace, Hologic, Vilvoorde, Belgium). The abdomen was visualized as a dorsal projection using X-ray imaging (29 kV, 40 mAs). X-ray imaging was possible throughout the whole experiment. In order to estimate the test meal fraction in the stomach, X-rays were taken every 5 or 10 minutes.
Image analysis

Images were processed using ImageJ software (ImageJ 1.43u, National Institute of Mental Health, Bethesda, Maryland, USA). After manually outlining the stomach, the software was used to calculate the surface area of the image of the stomach. The surface area directly after gavage of the meal (t=0) was set at 100% and GE was expressed as the fraction of the original stomach area over time.

Statistical analysis

Gastric accommodation. Results are expressed as mean ± SEM. Maximal IGP increase from baseline and areas under the curve (AUC) were calculated and compared using ANOVA and Tukey post test. Comparisons in experiments with L-NAME, atropine and vagotomy were made with Mann-Whitney U test. A p-value of less than 0.05 was considered statistically significant.

Gastric emptying. Mixed model analysis was performed using proc mixed in SAS 9.3 (SAS Institute, Cary, NC, USA), with fraction of the meal present in the stomach as the dependent variable and ‘condition’ (within-subject, 4 levels: water-vehicle (WV), water-PP (WPP), nutrient-vehicle (NV) & nutrient-PP (NPP) and ‘time’ (within-subject, 12 time points after meal) as independent categorical variables. Post-hoc t-test with Bonferroni correction for multiple testing was used for further exploring significant main and/or interaction effects.
**Results**

*IGP during nutrient infusion and influence of PP, L-NAME, atropine and vagotomy*

Nutrient infusion caused the IGP to increase, after which it stabilized, despite further nutrient infusion (Figure 1). The maximal pressure increase during control experiments was 6.2±0.4 mmHg. During nutrient infusion, rats receiving PP displayed a higher IGP increase compared to vehicle treated animals (maximal IGP increase of 8.1±0.5 mmHg and 9.1±0.9 mmHg for 33 and 100 pmol kg\(^{-1}\) min\(^{-1}\) PP respectively; Figure 1A). This effect was significant in the high-dose group (p<0.05), but not in the low-dose group (p>0.05). Similarly, AUC was only significantly different between placebo and 100 pmol kg\(^{-1}\) min\(^{-1}\) PP (p<0.005).

L-NAME administration significantly increased IGP during nutrient infusion compared to vehicle, with a maximal IGP increase of 11.2±1.2 mmHg (p<0.005; Figure 1B). Also AUC was different (p<0.005).

Atropine treatment resulted in an IGP increase, reaching 11.2±1.0 mmHg, which was significantly higher compared to vehicle (p<0.005; Figure 1C).

In vagotomized animals, the IGP increase during nutrient infusion tended to be less pronounced compared to controls (p=0.059), but maximal IGP increase did not differ (6.6±0.5 mmHg in controls vs. 6.2±0.5 mmHg in vagotomy; p>0.5; Figure 1D).

*Possible mechanisms of action of PP*

In the presence of L-NAME, the effect of PP was abolished, with IGP increasing up to 10.4±1.0 mmHg from baseline (p>0.5; Figure 2B). AUCs between L-NAME and L-NAME+PP did not differ (p>0.5; Figure 2A).

In the presence of atropine, PP did not induce a significant further increase in IGP (maximal IGP increase of 12.8±1.3 mmHg; p>0.1; Figure 2D). Similarly, AUCs between atropine and atropine+PP did not differ significantly (p>0.05; Figure 2C).
In vagotomized rats, IGP was increased during PP treatment compared to controls (p<0.005; Figure 2E). In addition, the maximal IGP after PP treatment was higher (8.5±0.8 mmHg vs 6.2±0.5 mmHg; p<0.5; Figure 2F).

Gastric emptying and influence of PP

Initial GE was fast (46.5 % emptied in the first 10 minutes), followed by a slower emptying rate (29.9 % emptied during the following 50 minutes). GE was reproducible since no difference could be observed between 2 consecutive control experiments (p>0.1, n=6).

Independently from caloric content, PP treatment resulted in a delay of GE (p<0.0001). This effect was observed in both nutrient (65.1±2.3 versus 53.2±2.1, p<0.005) and non-nutrient condition (51.4±2.5 versus 39.9±2.3, p<0.05). Furthermore, a significant effect of nutrient versus non-caloric was found in both the PP (65.1±2.3 versus 51.4±2.5, p=0.001) and vehicle condition (53.2±2.1 versus 39.9±2.3, p=0.007; Figure 3).
Discussion

We used IGP measurement during intragastric nutrient infusion as an assessment of GA in conscious rats. We investigated whether cholinergic, nitrergic and vagal pathways mediated the effect of PP. In addition we used X-ray to determine the effect of PP on GE.

Nutrient infusion initially caused an increase of IGP. This increase was followed by a plateau phase during which IGP hardly increased despite further nutrient infusion. These findings correspond to results from previous experiments, in which the same animal model was used [15]. It was suggested that the plateau phase, during which IGP is stable but intragastric nutrient infusion continues, represents a reflex gastric relaxation upon food ingestion and thus GA.

Many studies have confirmed the importance of NO as a mediator in gastric motility and also GA. Indeed, Lefebvre et al. showed that L-NAME reduced IGP decrease during vagal nerve stimulation in anesthetized rats, indicating that NO release is crucial for this response [21]. Another group showed that spontaneous antral relaxations were abolished by L-NAME [12]. In gastric fundus strips, electrical field stimulation-induced relaxation was inhibited by L-NAME [10]. In our study NOS inhibition resulted in a higher IGP increase during nutrient infusion compared to vehicle and this observation indicates that GA in our model is also mediated by NO.

Also muscarinic acetylcholine receptors have been shown to be involved in gastrointestinal motility [26]. We observed that during atropine treatment, GA was impaired. This finding is a confirmation of previous findings [15] and suggests that GA in rats is mediated through muscarinic receptor activation and that antagonism of the receptor causes an impaired accommodation response. Outcomes in studies that have investigated the role of cholinergic pathways in gastric motility and accommodation are however not consistent. In Sprague-Dawley rats, atropine had no effect on gastric volume response [29]. In dogs, gastric motility was decreased upon cholinergic blocking [7]. In humans, atropine enhanced gastric accommodation [22].

Denervation of the stomach was performed by means of subdiaphragmal vagotomy. In vagotomized animals the pattern of GA during nutrient infusion seemed to be
altered compared to outcome in non-vagotomized animals, but the GA reflex was still observed. It is generally accepted that GA is mediated by vagal pathways. Denervation of the stomach using capsaicin treatment has been reported to lead to an impaired accommodation response after intragastric saline infusion [37]. However, compensatory mechanisms after vagotomy have been described [1, 13], and such a mechanism may also have developed in our rat model.

We expected an inhibited gastric accommodation response in vagotomized animals. Although we did not see a clear difference in accommodation, IGP patterns were in fact different and this might indicate that comparisons of AUC and maximal IGP increase are insufficient to detect more subtle effects. As seen in supplementary figure 2, immediately after the start of nutrient infusion, IGP seems to increase faster in vagotomized rats. From previous experiments, we suggested that the phase during which IGP stabilized reflects accommodation. Apparently, the time period between nutrients starting to enter the stomach and reaching a stabilized IGP might also be relevant. Additional comparison of the AUC during the first 4 minutes of nutrient infusion (2 ml infused) showed that, compared to controls, IGP increase is significantly faster after vagotomy (p=0.0055; data not shown). This effect might be attributed to the fact that mechanoreceptors fail to efficiently signal to the brain in order to induce gastric relaxation, indicating that gut motility is disturbed, as would be expected [37].

During L-NAME treatment PP failed to further increase the IGP response during nutrient infusion. Although a ceiling effect cannot entirely be excluded, this seems very unlikely, as even higher IGPs were observed in the atropine experiments compared to during L-NAME treatment. Our observations thus suggest that the effect of PP involves a nitrergic pathway, i.e. inhibits NO release. To our knowledge, to date literature investigating whether PP acts on NO release is lacking, and the underlying mechanism deserves further analysis beyond the scope of the current study.

In the presence of atropine, PP failed to cause an enhanced IGP response, indicating that the mechanism of action of PP also involves muscarinic receptor activation. Atropine has been shown to abolish the PP-induced increase in antral motility in anesthetized rats [26].
It is suggested that PP acts via the vagal nerve, as Y₄-receptors are located in the dorsal vagal complex, which comprises the vagal lower motor neurons [39]. McTigue and Rogers found that bilateral cervical vagotomy diminished the PP-induced increased antral motility [26]. We observed that after vagotomy PP was still able to increase IGP. A possible explanation for this effect could be the beforementioned compensatory mechanisms that might have developed [1, 13]. It could also be possible that vagal-independent mechanisms contribute to the effect of PP. Banks et al. showed that PP could penetrate the blood brain barrier, suggesting that PP can exert its effect despite vagal disruption [4].

PP-overexpressing mice as well as mice receiving PP treatment display a decreased GE rate [3, 20]. Schmidt et al. showed that in healthy subjects GE of a solid meal was delayed after PP administration, but emptying of water was unaffected [32]. We however demonstrate that PP was able to slow down GE of both a non-caloric meal and a caloric nutrient meal in rats. A possible difference between our study and the one of Schmidt that might explain the observed difference is the dose of PP that was administered. Even though it is difficult to compare doses between humans and animals, we can state that the dose we used in rats was rather high. The dose used by Schmidt was relatively low (2.25 pmol kg⁻¹ min⁻¹), as there are reports from other studies administering higher doses to humans (up to 10 pmol kg⁻¹ min⁻¹) [5, 17]. This dose might have been too low to induce a delay in GE of water. The use of X-rays over the commonly used phenol red method has some advantages. There is no need to sacrifice animals; hence far less animals are required to acquire results. In addition, multiple observations per animal are possible, allowing paired comparisons. Disadvantages include the lack of three-dimensional imaging and the radioactive load.

Numerous studies in animals have demonstrated that PP is involved in food intake [2, 3]. Also humans reduce food intake upon PP, accompanied by lower hunger scores [5]. In healthy volunteers, pharmacologically-induced impaired GA was associated with early satiation and reduced food intake [16]. Impaired accommodation and early satiety were also correlated in functional dyspepsia patients [36]. Our findings fit in this context that PP has a two-fold effect. By inhibiting GA by increasing IGP, gastric mechanoreceptors are activated and lead to feelings of fullness [8]. In addition, Y₄-receptor stimulation in the brain leads to the inhibition of neuropeptide Y release and
hence the sensation of satiation, which eventually decreases food intake [3]. Interestingly, a number of studies in animals have shown enhanced feeding behavior after intra-cerebroventricular administration of PP [2, 9, 14]. Additional experiments investigating the receptor involved in this effect, have generated controversial outcomes [18]. Some authors have even suggested that a yet unknown receptor with affinity for PP might be involved in the increased food intake evoked by centrally administered PP. Clearly, more research is required to address this hypothesis.

PP has low affinity for the Y₁- and the Y₅-receptor, but it displays high affinity for Y₄-receptors, located in the area postrema in the dorsal vagal complex [30]. It has been shown that Y₄-receptor activation can alter gastrointestinal functions such as gastric motility, gallbladder contraction and pancreatic exocrine secretion. Y₄-receptors are also expressed on gastric smooth muscle [28]. Activation of these Y₄-receptors stimulates inositol triphosphate, which in turn increases intracellular Ca²⁺ levels, eventually resulting in contraction, which might result in an increase in pressure. In mice with a knock out of the Y₄-receptor, food intake is reduced and body weight is lowered [31]. Up to date, selective and potent Y₄-receptor antagonists are lacking, which hampers the study of the exact mechanism of action of PP.

In conclusion, PP impaired GA in conscious rats. The mechanism of action of PP involved the release of NO and the activation of the muscarinic receptor. After vagotomy PP still affected IGP, indicating a local peripheral mechanism of action. PP also delayed GE of both a non-caloric and a caloric meal. These findings might provide an alternative explanation for the effect of PP on food intake.
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Figure legends

Figure 1. Effect on IGP during intragastric nutrient infusion during i.v. administration of A) PP (33 and 100 pmol/kg/min); B) L-NAME (180 mg/kg/h); C) atropine (3 mg/kg/h); and D) subdiaphragmal vagotomy. Data are represented as mean ± SEM.

Figure 2. Effect of PP (100 pmol/kg/h) on IGP during intragastric nutrient infusion during i.v. administration of: A&B) L-NAME (180 mg/kg/h); C&D) atropine (3 mg/kg/h); and E&F) subdiaphragmal vagotomy. Data are represented as mean ± SEM.

Figure 3. Effect of PP (100 pmol kg⁻¹ min⁻¹) and caloric density on gastric emptying. Data are represented as mean ± SEM.
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