High gastrin cell activity and low ghrelin cell activity in high-anxiety Wistar Kyoto rats

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Abstract

Ghrelin is produced by gastric A-like cells and released in response to food deprivation. Interestingly, psychological stress also raises circulating ghrelin levels. This study compared plasma ghrelin levels in Sprague–Dawley (SPD) rats and high-anxiety Wistar Kyoto (WKY) rats. The two strains were also compared with respect to plasma gastrin, a gastric hormone with a pre- and postprandial release pattern opposite to that of ghrelin, and to the activity of the gastrin-dependent, histamine-forming ECL cells in the gastric mucosa. The rats were killed after being freely fed or after an over-night fast. The stomachs were weighed and tissue samples were collected for histological and biochemical analysis. Plasma ghrelin and gastrin levels were determined by RIA. While fasted SPD rats had higher plasma ghrelin

levels than fasted WKY rats ($P < 0.001$), plasma ghrelin did not differ between freely fed rats of the two strains. Gastrin levels were higher in fed WKY rats than in fed SPD rats ($P < 0.001$). Despite the higher plasma gastrin level, the oxyntic mucosal histidine decarboxylase (HDC) activity (a marker of ECL-cell activity) in fed rats and the mucosal thickness did not differ between the two strains. In a subsequent study, rats were subjected to water-avoidance stress for 60 min, causing plasma gastrin to increase in WKY rats ($P < 0.001$) but not in SPD rats. In conclusion, high-anxiety WKY rats had lower circulating ghrelin and higher gastrin than SPD rats in both the fasted and fed state, while the ECL-cell activity (HDC activity) was only moderately affected.

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Introduction

Wistar Kyoto (WKY) rats display more anxiety in behavioural tests than other rat strains (Paré 1992, Glowa & Hansen 1994). By comparison, Sprague–Dawley (SPD) rats represent a low-anxiety strain. Indeed, WKY rats appear to be particularly susceptible to stressful stimuli, manifested as stress-induced gastric ulcer and stress-evoked intestinal mucosal barrier dysfunction (Paré 1990, Saunders *et al.* 1997). WKY rats also display impaired gastric accommodation and visceral hypersensitivity in response to colorectal distension, perhaps due to high responsiveness to stress (Gunter *et al.* 2000, Nielsen *et al.* 2006).

The two peptide hormones gastrin and ghrelin are produced in the stomach (for a review see Walsh 1994, Kojima *et al.* 1999). While gastrin is produced by G cells in the antrum mucosa, ghrelin is produced by the A-like cells (ghrelin-immunoreactive cells; Date *et al.* 2000, Dornonville de la Cour *et al.* 2001, Rindi *et al.* 2002), located mainly in the oxyntic mucosa together with the histamine-producing ECL cells (Håkanson *et al.* 1994). ECL cells operate under the control of gastrin (Håkanson *et al.* 1994), while they are not affected by ghrelin (Dornonville de la Cour *et al.* 2004). Ghrelin cell-activity does not appear to be controlled by

gastrin (Dornonville de la Cour *et al.* 2001) while a role of ECL cells in the regulation of ghrelin cells has not been studied to our knowledge. G cells in the antrum are not regulated by ghrelin (Dornonville de la Cour *et al.* 2004), but are indirectly linked to ECL-cell activity since reduced activity of ECL cells reduces gastric acid secretion, which in turn increases the activity of G cells (for review, see Håkanson *et al.* 1994).

Gastrin and ghrelin have opposing secretory patterns; circulating ghrelin levels are high during fasting and decrease in response to food intake (Tschöp *et al.* 2000, Ariyasu *et al.* 2001, Dornonville de la Cour *et al.* 2001), while gastrin levels are low during fasting and high after food intake (for review, see Walsh 1994).

Gastrin stimulates gastric acid secretion by activating the ECL cell–parietal cell axis (for a review see Lindström *et al.* 2001). In addition, gastrin has tropic effects on the oxyntic mucosa (Håkanson *et al.* 1986). Ghrelin is thought to contribute to the regulation of food intake (Wren *et al.* 2000, Asakawa *et al.* 2001) and utilization of food (Tschöp *et al.* 2000, 2000) but its role in relation to gastrointestinal physiology is unclear. Ghrelin was at first claimed to stimulate gastric acid secretion after both systemic (Masuda *et al.* 2000) and central administration (Date *et al.* 2001) to anaesthetized

rats. In conscious rats, ghrelin was claimed to inhibit acid secretion upon systemic as well as central administration (Sibilia *et al.* 2002). In contrast, we showed recently that i.v. and s.c. infusion of ghrelin had no effect on acid secretion in conscious rats (gastric fistula rats and pylorus-ligated rats), and that it also had no effects on the different endocrine cells in the stomach (Dornonville de la Cour *et al.* 2004). However, it seems undisputed that exogenous ghrelin has a prokinetic effect on the stomach in rodents, manifested as an increased rate of gastric emptying (Masuda *et al.* 2000, Dornonville de la Cour *et al.* 2004). Interestingly, exogenous ghrelin enhances gastric emptying in patients with idiopathic gastroparesis (Tack *et al.* 2005). The physiological relevance of these findings is not clear, since genetically induced ghrelin deficiency does not affect gastric emptying (De Smet *et al.* 2006) and since administration of relatively low, but physiologically active doses of ghrelin had minimal effects on gastric motility in man (Cremonini *et al.* 2006).

Recent data demonstrate that circulating ghrelin levels are raised in response to psychological stress in both SPD rats and WKY rats (Kristensson *et al.* 2006), and that ghrelin expression in the oxyntic mucosa is increased in response to stressful stimuli such as tail pinch (Asakawa *et al.* 2001) and water immersion (Brzozowski *et al.* 2004). In addition, ghrelin produces anxiety-like behaviour in rats (Carlini *et al.* 2001, 2004). Interestingly, obestatin, a recently discovered peptide hormone derived from the same precursor as ghrelin, is claimed to produce opposing effects on ingestive behaviour and cause anxiolytic effects in rats (Zhang *et al.* 2005, Carlini *et al.* 2007). Since circulating ghrelin is elevated in response to stress, we compared SPD rats and high-anxiety WKY rats with respect to the activity of A-like cells and G cells. We measured plasma ghrelin, which reflects A-like cell activity (Ariyasu *et al.* 2001, Dornonville de la Cour *et al.* 2001, Jeon *et al.* 2004) and plasma gastrin, which reflects G-cell activity. We also examined the activity of the ECL cells in the two strains since these cells are the main target for gastrin (Håkanson *et al.* 1994). This was done by measuring the activity of oxyntic mucosal histidine decarboxylase (HDC), a well-known marker of ECL-cell activity (Håkanson *et al.* 1974). Finally, we examined circulating gastrin after 60 min of water-avoidance stress in order to see whether stress influences not only circulating ghrelin but also gastrin.

Materials and Methods

Animals

From Harlan (Horst, The Netherlands), 41 SPD and 42 WKY rats (females) were purchased. WKY rats were 12–14 weeks of age, while SPD rats were 10–12 weeks of age at the start of experiments. They were acclimatized to the animal facility at AstraZeneca for at least 1 week after arrival. They were housed six in each cage ($h=40$ cm, $w=80$ cm, $l=60$ cm) and kept in a climate-controlled room on a 12 h

light:12 h darkness cycle. Food, water and building material were supplied at all times except during fasting when food and building material were withdrawn. All experiments were approved by the local ethics review committee on animal experiments in Göteborg, Sweden.

Comparison of WKY and SPD rats

While 18 SPD rats and 18 WKY rats had free access to food, 18 SPD rats and 18 WKY rats were fasted for 16–18 h overnight. All rats were killed at the same time, in the morning between 0800 and 1000 h. Blood samples were collected by exsanguination from the heart for measurement of plasma ghrelin and plasma gastrin concentrations. The stomachs were removed, cut open along the major curvature from the pyloric sphincter to the oesophagus, emptied and weighed. Small tissue samples were collected from the acid-producing part of the stomach (fundus) for assessment of mucosal thickness (for details see Histology). In five fasted and six fed SPD rats and six fasted and fed WKY rats, the oxyntic mucosa was scraped-off the stomach wall and stored at -20°C until the determination of HDC activity (see Biochemical analysis).

Water avoidance stress

Six WKY rats and five SPD rats (fed *ad libitum*) were subjected to water avoidance stress (see below), applied as a psychological stressor (Enck *et al.* 1989). Thirty minutes before exposure to the stress, the rats were anaesthetized with isoflurane (Forene, Abbott Pharmaceutica) and blood was drawn from the tail and collected in heparinized tubes. The rats were allowed to wake up before being placed on a 9.5 cm high platform ($\varnothing=7.9$ cm, the platform itself is 1 cm high) in a transparent cage ($h=37$ cm, $w=23$ cm, $l=38$ cm), containing water. The water had a temperature of $18\pm 1^{\circ}\text{C}$ and reached the lower edge of the platform. All experiments were performed between 0800 and 1000 h. The rats were kept in the cages for 1 h. A second blood sample was drawn from the heart (exsanguination) during renewed isoflurane anaesthesia. Plasma was collected and stored at -20°C until further analysis.

Histology

Small tissue samples (2×4 mm) were taken from the central part of the fundus (along the major curvature) and immersed in formalin (10%) for 2–3 days before transfer to 0.01 M PBS and to 70% ethanol a few days later. The tissue samples were embedded in paraffin, sectioned at 4 μm thickness, perpendicular to the mucosal surface, and placed on glass slides. Only transverse sections were examined (entire thickness of mucosa visible). The mucosal thickness was determined after deparaffinizing the sections in xylene and washing in distilled water before staining with hematoxylin/eosin. The mucosal thickness was measured from the base of the gastric glands to

the mucosal surface, three measurements per section, one section per animal.

Biochemical analysis

Histidine decarboxylase activity The oxyntic mucosa was weighed and stored at -20°C until homogenization in ice-cold 0.1 M sodium phosphate buffer, pH 7.4, to a concentration of 100 mg/ml. Aliquots (80 μl) of the homogenates were incubated with L-[^{14}C]histidine (specific activity 50 mCi/mmol), 0.5 mM L-histidine and 0.01 mM pyridoxal-5-phosphate in a total volume of 160 μl at 37°C for 1 h as described previously (Larsson *et al.* 1986). The HDC activity was expressed as pmol $^{14}\text{CO}_2$ /mg per h.

Ghrelin A commercially available RIA kit (Phoenix Pharmaceuticals, Belmont, CA, USA) was used to determine ghrelin according to the manual supplied by the manufacturer. Plasma samples were diluted 1:4 or 1:10 before the analysis. The antiserum was raised against octanoylated human ghrelin; the tracer was radioiodinated (I^{125}) ghrelin-28. Rat ghrelin-28 was used as standard. The antiserum recognizes both octanoylated and des-octanoylated ghrelin-28. The intra- and inter-assay variation was 3 and 8% respectively. The concentration of ghrelin in plasma was expressed as picomole equivalents of rat ghrelin-28 per litre.

Gastrin Plasma was analyzed for gastrin by RIA as described earlier using rat gastrin-17 (Research Plus, South Plainfield, NJ, USA) as standard. The antiserum (no. 2604, a kind gift from Professor J F Rehfeld, Rigshospitalet, Copenhagen, Denmark) was raised against the 2–17 fragment of human gastrin-17 and is specific for the bioactive C-terminus. It binds gastrin-34 and gastrin-17 with the same potency. The intra- and inter-assay variation was 7 and 8% respectively. The gastrin concentration was expressed as picomole rat gastrin-17 equivalents per litre plasma.

Statistical analysis

Statistical analysis was performed using Student's unpaired *t*-test followed by corrections for multiple comparisons using Bonferroni. A *P* value <0.05 was considered statistically significant. All results are expressed as means $\pm$ S.E.M.; *n* reflects the number of individual rats.

Results

General features of SPD rats and WKY rats

The WKY rats in the current study were slightly older than the SPD rats (Table 1). Nonetheless, the WKY rats weighed less ($P<0.01$), as did their stomachs ($P<0.001$). Oxyntic mucosal thickness did not differ between the two strains (Table 1).

Table 1 Comparison between Sprague–Dawley (SPD) and Wistar Kyoto (WKY) rats with respect to age, body weight, stomach weight and oxyntic mucosa thickness

	SPD	WKY	<i>n</i>
Parameter			
Age (weeks)	10–12	12–14	24
Body weight (g)	213 ± 3	$199 \pm 3^*$	24
Stomach weight (g)	1.34 ± 0.03	$1.04 \pm 0.02^+$	24
Stomach weight/body weight (%)	0.63 ± 0.01	$0.52 \pm 0.01^+$	24
Oxyntic mucosal thickness (μm)	581 ± 24	560 ± 18	12

Age, body weight and gastric parameters are given for 24 rats of each strain. Additional rats were used for stress experiments. * $P<0.01$, $^+P<0.001$.

Ghrelin-cell activity

The circulating ghrelin concentration in fasted SPD rats was more than twice that of the fed controls ($P<0.001$, Fig. 1A). In WKY rats, fasting increased circulating ghrelin by only 40% ($P<0.01$). Fasted SPD rats had much higher ghrelin levels than fasted WKY rats ($P<0.001$), and fed SPD rats tended to have higher ghrelin levels than fed WKY rats (by 20%), but this difference was not statistically significant.

Gastrin-cell activity

As expected, circulating gastrin levels were high in fed rats and low after an overnight fast in both strains of rats ($P<0.001$, Fig. 1B). However, the gastrin levels were twofold higher ($P<0.001$) in fed WKY rats than in fed SPD rats and almost threefold higher in fasted WKY rats than in fasted SPD rats, albeit the latter difference was not statistically significant ($P=0.06$).

ECL-cell activity

As expected, the oxyntic mucosal HDC activity was higher in fed than in fasted rats ($P<0.001$, see also Håkanson *et al.* 1994; Fig. 1C). WKY rats had higher HDC activity than SPD rats in the fasted state ($P<0.001$) and tended to have higher activity in the fed state.

Effects of psychological stress on gastrin levels

In accordance with the results shown in Fig. 1B, gastrin levels were higher in fed WKY rats than in fed SPD rats ($P<0.001$, Table 2). While water-avoidance stress failed to affect circulating gastrin in the SPD rats, it raised it in the WKY rats ($P<0.001$).

Discussion

The current study demonstrates that high-anxiety WKY rats display high G-cell activity and low A-like (ghrelin) cell activity in comparison to SPD rats, which are classified as a low-anxiety

Figure 1 (A) Plasma ghrelin ($n=12-18$), (B) plasma gastrin ($n=10$) and (C) oxyntic mucosal histidine decarboxylase activity ($n=5-6$) in fed and fasted SPD and WKY rats. Statistical analysis was performed using Student's t -test followed by correction for multiple comparisons. Mean $\pm$ S.E.M. ** $P<0.01$, *** $P<0.001$.

Table 2 Effects of water-avoidance stress (WAS) on plasma gastrin levels in fed Sprague-Dawley (SPD) and fed Wistar Kyoto (WKY) rats

	Plasma gastrin before WAS (pmol/l)	Plasma gastrin after WAS (pmol/l)	n
Strain			
SPD	87.5 $\pm$ 8.2	83.3 $\pm$ 16.2	5
WKY	156.1 $\pm$ 5.9*	225.2 $\pm$ 8.2*.*	6

*Plasma gastrin levels in WKY rats after WAS were significantly ($P<0.001$) higher than in SPD rats after WAS. *Plasma gastrin levels in WKY rats were significantly ($P<0.001$) higher than in SPD rats. *Plasma gastrin levels in WKY rats after WAS were significantly ($P<0.001$) higher than before WAS.

strain. The activity of a third gastric endocrine cell type, the ECL cells, is reflected in the HDC activity; it was moderately increased in fasted WKY rats compared with SPD rats.

Circulating ghrelin levels are known to increase upon food withdrawal (Tschöp *et al.* 2000, Ariyasu *et al.* 2001, Dornonville de la Cour *et al.* 2001). Metabolic stimuli (or the lack of such stimuli) seem to be paramount in the regulation of ghrelin release (Qader *et al.* 2005, Dornonville de la Cour *et al.* 2006). We have recently demonstrated that water-avoidance stress increases ghrelin levels in both SPD and WKY rats (Kristensson *et al.* 2006) and recent reports indicate that local microinfusion of adrenaline/noradrenaline into the gastric submucosa (Dornonville de la Cour *et al.* 2007) and direct stimulation of the sympathetic nervous system (Mundinger *et al.* 2006) stimulates ghrelin secretion. Thus, A-like cells may release ghrelin also in response to stimuli other than those associated with reduced nutritional status. Previously, fed WKY rats were observed to have 25% lower plasma ghrelin levels than the SPD rats although this difference was not statistically significant (Kristensson *et al.* 2006). Also the results of the present study suggest that fed WKY rats have somewhat lower plasma ghrelin levels than SPD rats (20% lower, not statistically significant). However, dramatic differences between the strains were seen after an overnight fast in that ghrelin increased more than twofold in SPD rats but only 40% in WKY rats. Our previous study revealed that although circulating ghrelin increased significantly in WKY rats in response to stress, the magnitude of the increase (40%) was lower than the increase seen in SPD rats (85%; Kristensson *et al.* 2006). Taken together, the results suggest that A-like cells in SPD rats are more sensitive to fasting-evoked and stress-evoked stimuli than A-like cells in WKY rats. Ghrelin is claimed to protect the stomach against stress-evoked gastric lesions (Brzozowski *et al.* 2004). Whether the susceptibility of WKY rats to stress-induced gastric ulcers is associated with suppressed activity of the A-like cells remains to be investigated.

Interestingly, circulating gastrin responded somewhat differently in the two strains. Although, as expected, food deprivation reduced plasma gastrin, circulating levels were more than twofold higher in WKY rats than in SPD rats in both the fasted and fed state. Gastrin levels in fed WKY rats

approached levels reached in SPD rats treated with proton pump inhibitors or histamine H₂ receptor antagonists (Håkanson *et al.* 1995). This observation is consistent with the observation that WKY rats have a lower rate of gastric acid secretion than SPD rats (M Astin, M Florentzson, B Holstem and K Andersson personal communication).

The main targets for gastrin are to be found in the oxyntic mucosa, where gastrin stimulates ECL-cell activity and exerts trophic effects (Håkanson & Sundler 1990). Surprisingly, given the high levels of circulating gastrin in WKY rats, the thickness of the oxyntic mucosa did not differ between the two strains. The activity of the ECL cells, reflected in the oxyntic mucosal HDC activity, is controlled by circulating gastrin (Håkanson *et al.* 1974). Although not statistically significant, the HDC activity tended to be lower in fed SPD rats than in fed WKY rats, suggesting that perhaps additional factors are required for gastrin to exert maximal effects in the oxyntic mucosa and that these factors may be lacking in WKY rats. Gastrin acts synergistically with the vagus in the trophic control of the rat oxyntic mucosa, and the ECL cells represent an important target for both gastrin and the vagus (Axelson *et al.* 1988, Norlén *et al.* 2005) indicating that they most likely also operate under nervous control.

Interestingly, we could show that the relatively high plasma gastrin levels observed in fed WKY rats were increased even further after subjecting the rats to psychological stress, while circulating gastrin remained unchanged in the SPD rats. Conflicting results exist regarding the effects of psychological stress on circulating gastrin levels. Paternico *et al.* (1994) showed that stressful stimuli (cold pressor test) did not affect circulating gastrin levels in man and Sandin *et al.* (1998) could not demonstrate an effect of stress on gastrin levels in horses. On the other hand, acoustic stress in dogs was found to enhance feeding-induced release of gastrin (Gue *et al.* 1989). Thus, in some species, psychological stress seems to be capable of mobilizing gastrin. The results of the present study extend these findings to the WKY rat strain which is considered hyper-responsive to stress.

In conclusion, our data show that G cells in the WKY rats are more active than those in the SPD rats, manifested as higher plasma gastrin levels, regardless of the prandial state, and as increased levels following stress. In contrast, A-like cells in WKY rats appear to be less active than A-like cells in SPD rats, manifested as low ghrelin levels in response to fasting (current study) and stress (Kristensson *et al.* 2006). Possible functional consequences of these different activity ratios of three of the major gastric endocrine cell types remain to be explored.

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