JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2013, 64, 6, 719-725
www.jpp.krakow.pl

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GASTRIN MEDIATED DOWN REGULATION OF GHRELIN AND ITS PATHOPHYSIOLOGICAL ROLE IN ATROPHIC GASTRITIS

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The gastric hormone ghrelin is known as an important factor for energy homeostasis, appetite regulation and control of body weight. So far, ghrelin has mainly been examined as a serological marker for gastrointestinal diseases, and only a few publications have highlighted its role in local effects like mucus secretion. Ghrelin can be regarded as a gastro-protective factor, but little is known about the distribution and activity of ghrelin cells in pathologically modified tissues. We aimed to examine the morphological changes in ghrelin expression under several inflammatory, metaplastic and carcinogenic conditions of the upper gastrointestinal tract. In particular, autoimmune gastritis showed interesting remodeling effects in terms of ghrelin expression within neuroendocrine cell hyperplasia by immunohistochemistry. Using confocal laser microscopy, the gastrin/cholecystokinin receptor (CCKB) could be detected on normal ghrelin cells as well as in autoimmune gastritis. Functionally, we found evidence for a physiological interaction between gastrin and ghrelin in a primary rodent cell culture model. Additionally, we gathered serological data from patients with different basic gastrin levels due to long-term autoimmune gastritis or short-term proton pump inhibitor treatment with slightly reactive plasma gastrin elevations. Total ghrelin plasma levels showed a significantly inverse correlation with gastrin under long-term conditions. Autoimmune gastritis as a relevant condition within gastric carcinogenesis therefore has two effects on ghrelin-positive cells due to hypergastrinemia. On the one hand, gastrin stimulates the proliferation of ghrelin-positive cells as integral part of neuroendocrine cell hyperplasia, while on the other hand, plasma ghrelin is reduced by gastrin and lost in pseudopyloric and intestinal metaplastic areas. Ghrelin is necessary for the maintenance of the mucosal barrier and might play a role in gastric carcinogenesis, if altered under these pre neoplastic conditions.

Key words: *ghrelin, gastrin, autoimmune gastritis, gastric cancer, Helicobacter pylori, gastrin/cholecystokinin receptor, enterochromaffin-like cells*

INTRODUCTION

The auto-regulatory gastric hormonal system is complex (*Fig. 1*). In brief, the physiological postprandial rise in luminal pH values in the gastric fundus leads indirectly to somatostatin-mediated increase of gastrin from the antral mucosa. In a feedback loop, the elevated gastrin levels directly influence the parietal cells in the gastric fundus, or affect enterochromaffin-like cells with subsequent histamine secretion. As a consequence, parietal cells are stimulated to secrete acid and contribute to the physiological restoration of low pH values in the stomach (1, 2). Disturbances in the homeostasis of gastric hormones play a major role in the development of atrophic gastritis, neuroendocrine tumors and adenocarcinomas, respectively (3).

With its discovery in 1999, ghrelin was introduced into this scenario as a new gastric hormone (4). Ghrelin is mainly located in the oxyntic mucosa of the gastric fundus and its expression is attributed to the neuroendocrine cell subtype of A-like cells (5). After gastrectomy, the levels of ghrelin decrease substantially (6). Various studies have investigated ghrelin and its systemic effects regarding growth hormone release from the pituitary gland, appetite regulation and its impact on body weight (7, 8). However, ghrelin is also a hormone with gastro-protective local effects. It stimulates propulsion and mucus secretion and contributes to the healing process after a mucosal injury. Therefore, ghrelin is essential for maintaining the mucosal barrier of the human stomach (9-12).

The purpose of this study was to clarify the biological mechanism and the role of ghrelin in the complex gastric hormonal system with special regards to interactions with gastrin. We tested alterations in the distribution and number of ghrelin cells in different diseases, examined the presence of the

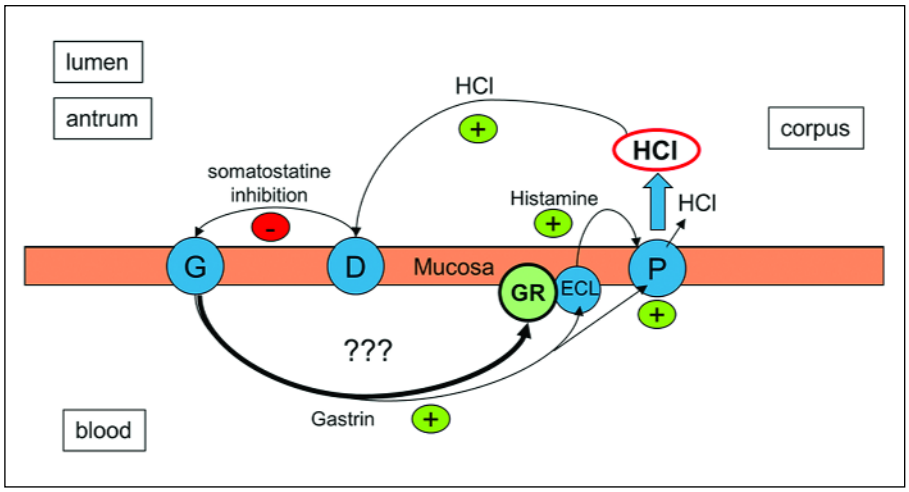

Fig. 1. Scheme of gastric hormones with feedback loops (+ stimulation, - inhibition). On the left: antral mucosa with D = somatostatin cells and G = gastrin cells. On the right: acid producing corpus and fundus mucosa with P = parietal cell, ECL = enterochromaffin-like cells and GR = ghrelin cells.

gastrin/cholecystokinin receptor (CCKB) on ghrelin-expressing cells and assessed the effects of gastrin on ghrelin expression *in vitro* and *in vivo*.

MATERIALS AND METHODS

Patient material

Human gastric biopsy specimens from different normal sites, different types of gastritis and metaplastic conditions were used as well as tissue sections from surgical gastric carcinoma specimens. A detailed overview of these samples is given in *Table 1*. All samples were taken from the diagnostic files of the Institute of Pathology of the University Hospital Erlangen. They were fixed in 4% buffered formaldehyde and embedded in paraffin wax (FFPE).

The patient cohorts for blood analysis comprised normal participants (n=18) as well as patients with post-surgical administration of proton pump inhibitors for 72 hours as a testing model for short-term elevated gastrin levels (n=18). From the group of patients with long-term autoimmune gastritis, only a few serological samples were available (n=8). The study design was approved by the local ethical committee (approval on 07/05/2010) and informed written consent was obtained from all participating patients. The study was performed in line with the declaration of Helsinki.

Histology and immunohistochemistry

Serial 3 m sections from FFPE tissue blocks were deparaffinized and stained with hematoxylin and eosin (H&E) following routine protocols. Ghrelin immunohistochemistry was performed as follows. After deparaffinizing, the sections were transferred to citrate buffer and pretreated in a pressure cooker/steamer for 90 seconds. After the sections had been treated with 10% hydrogen peroxide for 15 minutes, they were incubated with a goat anti-ghrelin antibody (1:500; sc-10368 Santa Cruz Biotechnology, Santa Cruz, USA) overnight at room temperature. Binding of the primary antibody was detected using a biotinylated rabbit-anti-goat antibody (1:100; Dako Denmark) for 30 min. The samples were incubated with a horseradish peroxidase complex (Dako, Glostrup, Denmark) for 30 min. Then, the sections were stained with AEC Substrate-Chromogen (3-amino-9-ethylcarbazole; Dako, Glostrup, Denmark) for 3 min. Counterstaining was accomplished using hematoxylin for 10 seconds. Omission of the primary antibody served as the negative control.

Double immunofluorescence

Sections of normal corpus and autoimmune gastritis were treated as mentioned above. The primary antibodies used were a goat anti-ghrelin antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, USA) and a mouse anti-chromogranin antibody (1:1,000; Beckman Coulter, Fullerton, USA). After overnight incubation, a Cy2-labeled donkey-anti-goat antibody (1:100) and a Cy3-labeled rabbit-anti-mouse antibody (1:100) were added for one hour. Using the same protocol, staining was also performed using a goat anti-ghrelin antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, USA) and a rabbit anti-gastrin/cholecystokinin B receptor antibody (1:100; GeneTex, San Antonio, USA). As secondary antibodies, a Cy2-labeled donkey-anti-goat antibody (1:50) and a Cy3-labeled goat-anti-rabbit antibody (1:50) were used. All fluorescently labeled secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, West Grove, USA. Nuclear counterstaining was performed with DAPI (1:5000).

Confocal images were acquired using a Bio-Rad MRC 1000 confocal laser scanning system (Bio-Rad, Hemel Hempstead, UK) equipped with a three-line krypton-argon laser (American Laser Corporation, Salt Lake City, USA) and attached to a Nikon Diaphot 300 (Nikon, Dusseldorf, Germany).

Rodent gastric primary cell culture

Mucosal cells from the stomachs of adult Wistar rats (n=4 per experiment; body weight 200–230 g; Charles River, Sulzfeld, Germany) were isolated using an everted sac technique which was slightly modified from Prinz *et al.* (13). Briefly, stomachs were turned inside out, filled with pronase E (1.2 mg/ml, Sigma-Aldrich, Munich, Germany) and alternatingly incubated for 30 minutes with sterile filtered Solution A (0.5 mM $NaH_2PO_4 \times H_2O$, 1.0 mM $Na_2HPO_4 \times 2\ H_2O$, 20 mM $NaHCO_3$, 70 mM NaCl, 5 mM KCl, 11 mM glucose, 50 mM HEPES, 0.7 mM EDTA, 10 mg/ml albumin, pH 7.8) and Solution B (0.5 mM $NaH_2PO_4 \times H_2O$, 1.0 mM $Na_2HPO_4 \times 2\ H_2O$, 20 mM $NaHCO_3$, 70 mM NaCl, 5 mM KCl, 11 mM glucose, 50 mM HEPES, 0.1 mM $CaCl_2$, 1.5 mM $MgCl_2 \times 6\ H_2O$, 5 mg/ml albumin, pH 7.4). The fluids were continuously aerated with 20% O_2/80% N_2. After two hours, solutions A and B were changed every 10 minutes under slight stirring and without aeration. Cells were collected, filtered and transferred to RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany).

Cells from the whole gastric mucosa were incubated in intervals of 0, 6, 12, 24 and 48 hours with pentagastrin, which is

Table 1. Investigated tissue samples.

Tissue	Ghrelin density	Ghrelin positivity	Gender (m:f)	Mean age (range)
Normal				
Oesophagus	–	0.0% (0/12)	6:6	19.0 (1–84)
Fundus	+++	91.7% (11/12)	7:5	51.2 (11–81)
Antrum	+	72.7% (8/11)	6:5	32.7 (1–66)
Duodenum	(+)	100.0% (11/11)	7:4	58.7 (34–83)
Inflammation				
Oesophageal reflux disease	–	0.0% (0/9)	7:2	46.9 (11–77)
H.pylori gastritis	++	100.0% (10/10)	4:6	63.9 (26–90)
Type-A atrophic gastritis	+/-	100.0% (20/20)	9:11	71.5 (47–84)
Metaplasia				
Barrett's mucosa	(+)	83.3% (10/12)	6:6	65.1 (22–82)
Intestinal metaplasia of the stomach	–	0.0% (0/20)	9:11	71.5 (47–84)
Gastric metaplasia of the duodenum	(+)	72.7% (8/11)	7:4	56.8 (43–81)
Gastric carcinoma	–	0.0% (0/26)	16:10	62.0 (39–83)
Intestinal type	–	0.0% (0/22)		
Diffuse type	–	0.0% (0/4)		

a homologue to human gastrin (Sigma-Aldrich, Munich, Germany) at concentrations of 0.0, 0.5, 2.5 und 5.0 µg/ml. Cell numbering was used to evaluate the toxicity of the gastrin levels used. All experiments were performed in triplicate. The German National Research Council's guidelines for the care and use of laboratory animals were followed and ethical approval was obtained for the project (TS-13/06, approval on 11/10/2006).

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

RNA from pentagastrin-treated primary rat stomach cells was isolated with the RNeasy micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The following rat specific primers were used for RT-PCR: ghrelin: forward, 5'- TTGAGCCCAGAGCACCAGAAAG-3'; reverse, 5'-GCCAACATCGAAGGGAGCAT-3'. GAPDH: forward, 5'-GCCAGCCTCGTCTCATAGACA-3'; reverse, 5'-TGGTAACCAGGCGTCCGATA-3' (Eurofins MWG, Ebersberg, Germany). GAPDH was used as the endogenous control. qPCR based on the SYBR Green technique was performed on a 7500 fast real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Relative gene expression levels were calculated using the ΔΔCT-method (14).

ELISA and RIA of gastrin and total ghrelin in patient plasma

Blood samples from the patient groups described above were collected from a peripheral vein under basal conditions, following at least 12 hours of fasting. Plasma was collected in aprotinin-EDTA vials for stabilization, separated the following day and stored at -20°C for subsequent investigation. A gastrin radioimmunoassay (RIA) was performed as described previously.(3) Total ghrelin (Spi-Bio, Montigny le Bretonneux, France) ELISAs were used to determine the plasma levels according to the manufacturer's instructions.

Statistical analysis

PASW/SPSS 19.0 (IBM, NY, USA) was used for statistical analysis. To determine significant differences between treated and untreated primary cell cultures and data from ELISA experiments, Student's t-tests for dependent and independent variables were used. To find significant associations between plasma ghrelin and gastrin levels, a Pearson correlation and the Kolmogorov-Smirnov test for a normal distribution were applied. Statistical significance was set at $p \leq 0.05$.

RESULTS

Ghrelin expression in gastritis, metaplasia and gastric carcinoma differs from the normal distribution pattern. As expected, the highest density of ghrelin-expressing cells was immunohistochemically found in the fundic mucosa of the stomach. Scattered ghrelin-positive cells were distributed between parietal and chief cells of the oxyntic mucosa (*Fig. 2a*), whereas the antral mucosa was ghrelin negative (*Fig. 2b*). In other areas of the upper gastrointestinal tract, at least singular ghrelin-positive cells could be perceived (*Table 1*). Only in the squamous epithelium of the normal esophagus were no ghrelin cells detectable, which was the same in the squamous epithelium in gastroesophageal reflux disease.

During *H. pylori*-mediated gastritis, the inflammatory cells expand from the top toward the bottom in the fundic mucosa and partially destroy ghrelin-expressing cells, which were decreased in number and pushed down toward the base of the fundic glands. In atrophic gastritis, this phenomenon was enhanced. Pseudopyloric glands, as well as intestinal metaplasia of the fundic mucosa were negative for ghrelin (*Fig. 2c*). Remarkably ghrelin expression was increased in areas with neuroendocrine cell hyperplasia and was found to be an unequivocal aspect of so-called neuroendocrine nodules. Other types of metaplasia in the upper gastrointestinal tract only showed slight differences in ghrelin expression (*Table 1*). Interestingly, gastric adenocarcinoma was associated with the elimination of gastro-protective ghrelin cells as a result of malignant overgrowth (*Fig. 2d*).

Ghrelin-positive cells contribute to neuroendocrine cell hyperplasia in atrophic autoimmune gastritis

To better understand the quantitative number of ghrelin cells within areas of neuroendocrine cell hyperplasia, specimens from atrophic autoimmune gastritis (*Table 1*) were analyzed by chromogranin/ghrelin double immunofluorescent staining. In the normal fundic mucosa, approximately 20% of all chromogranin-positive neuroendocrine cells co-expressed ghrelin (*Fig. 3a*). In autoimmune gastritis, ghrelin-expressing cells comprised nearly half of the investigated neuroendocrine

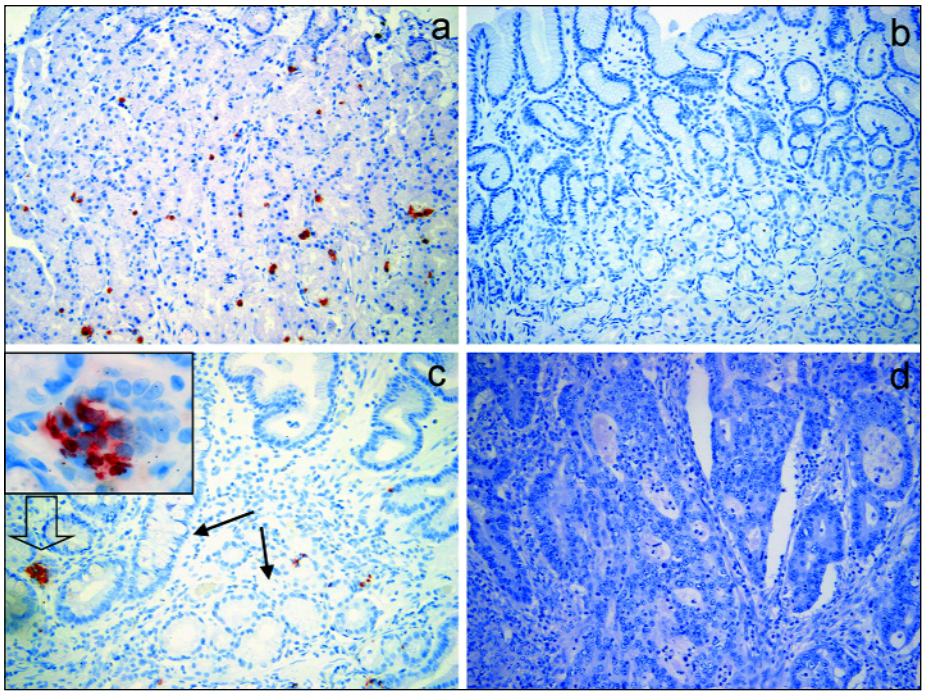

Fig. 2. Ghrelin immunohistochemistry (200× magnification). Ghrelin cells are highlighted in red-brown. The physiologic condition shows dense singular interspersed ghrelin-positive cells between the fundic glands of the normal corpus mucosa (*a*), whereas normal antral glands are negative (*b*). Autoimmune gastritis (*c*) of the gastric corpus shows remarkable remodeling in comparison to the normal corpus mucosa (*a*). Arrows in (*c*) indicate ghrelin-negative areas of intestinal metaplasia and pseudopyloric glands, whereas endocrine hyperplasia is positive for ghrelin (400× magnification). Gastric adenocarcinoma (*d*) is negative for ghrelin.

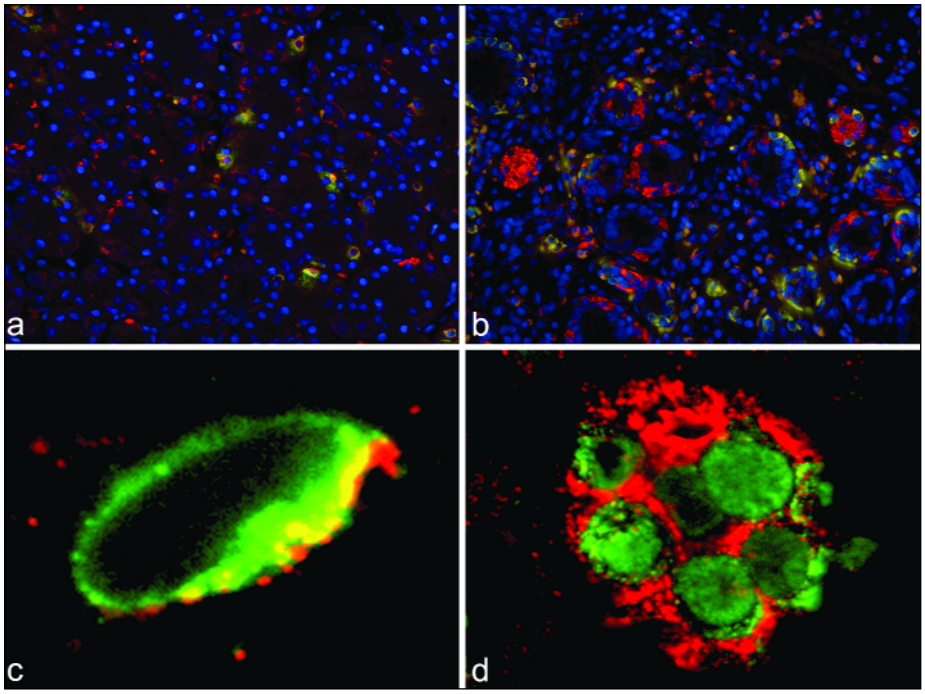

Fig. 3. In the upper part, double immunofluorescent staining for ghrelin (green) and chromogranin (red) (*a*, *b*). Note that ghrelin cells are all part of the chromogranin pool of neuroendocrine cells, resulting in the merged yellow color for ghrelin positive cells. In the lower part, double immunofluorescent staining for ghrelin (green) and the gastrin/cholecystokinin (CCKB) receptor (red) (*c*, *d*). Note the expected membranous red CCKB receptor staining encircling the green cytoplasmic ghrelin staining. Again, the normal corpus (*a*, *c*) and autoimmune gastritis (*b*, *d*) of the corpus were compared.

cell clusters identifiable within the corresponding typical linear and nodular chromogranin staining pattern.

The gastrin/cholecystokinin receptor (CCKB) is colocalized on human ghrelin-positive cells

The development of neuroendocrine cell hyperplasia is mainly influenced by elevated gastrin levels. As a first step, we investigated whether the gastrin/cholecystokinin receptor (CCKB) is co-expressed on ghrelin-expressing cells. In normal gastric mucosa, we could confirm faint membrane-localized staining of this receptor on ghrelin-positive cells, indicated by red dots around the intensely green stained ghrelin-expressing cell (*Fig. 3c*). This expression profile was even stronger in atrophic autoimmune gastritis, where the arrangement in neuroendocrine cell clusters enhanced the red areas of gastrin/cholecystokinin receptor (CCKB) staining on the cell surface of ghrelin-positive cells (*Fig. 3d*).

Ghrelin is down-regulated in primary gastric cells during gastrin stimulation

As a functional model, we chose primary gastric cells from rats. This model excludes systemic compensatory effects and focuses on the local effects of elevated gastrin levels. The incubation of primary gastric cells with pentagastrin showed a significant ($p<0.015$) and dose-dependent down-regulation of ghrelin mRNA by pentagastrin, down to 43.5% of its initial

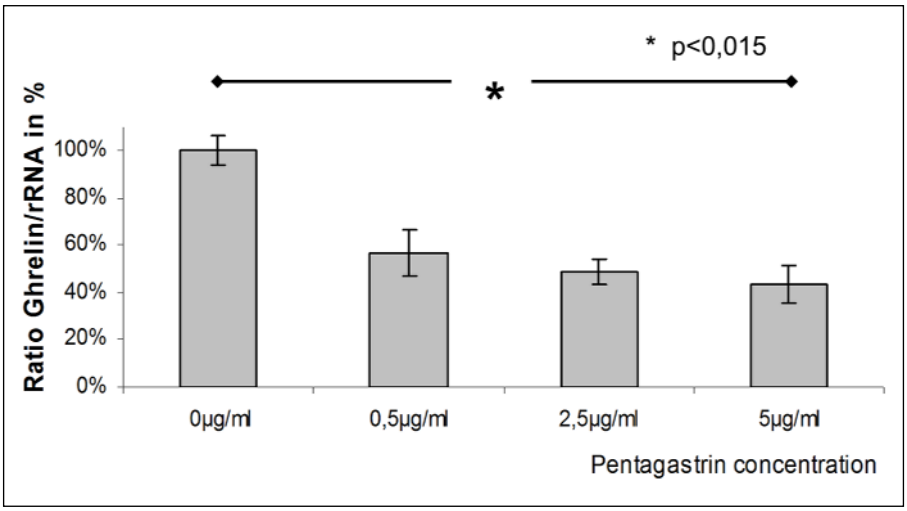

Fig. 4. Dose-dependent transcriptional silencing of ghrelin by pentagastrin stimulation in a primary rodent cell culture model. Pentagastrin was used as a strong and stable gastrin analogue. Maximal reduction could be exerted down to 43.5% in comparison to untreated samples (quantitative RT-PCR, $\Delta\Delta C_t$-method).

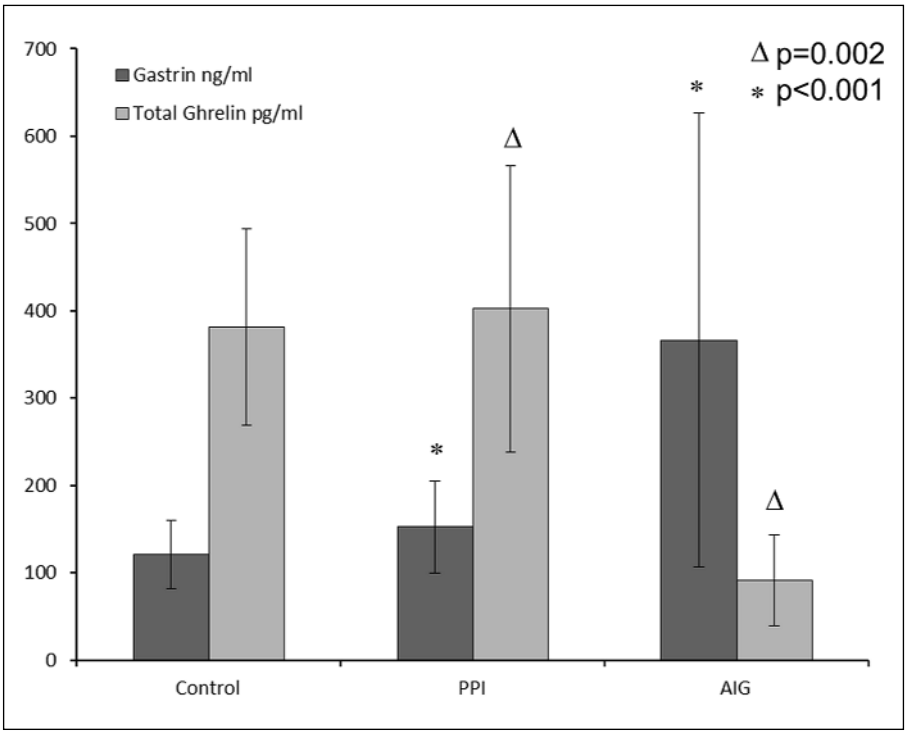

Fig. 5. Preprandial serological data for gastrin and total ghrelin from patients with a normal stomach mucosa, short-term proton pump inhibitor-treated patients and from patients with autoimmune gastritis. Data are shown as mean with the standard deviation (unpaired Student's t-test).

expression (*Fig. 4*). Cell counting of treated versus untreated samples showed no elevated toxicity at the pentagastrin doses used in this study.

Plasma levels of ghrelin are affected by long-term gastrin elevation

Transferring these experimental data into the clinical context, we next investigated ghrelin plasma levels under different conditions. We evaluated the short-term increase in gastrin following intake of proton pump inhibitors versus the long-term gastrin increase in the situation of autoimmune gastritis in comparison to levels normal patients. Total ghrelin and total gastrin values showed a significantly inverse correlation (Pearson correlation, coefficient –0.516, $p<0.001$). However, only the strong and long-term 3.0-fold increase in gastrin seen in autoimmune gastritis repressed ghrelin secretion significantly by a factor of 4.2 (*Fig. 5*).

DISCUSSION

Physiological ghrelin expression in the normal mucosa of the upper gastrointestinal tract was mainly restricted to the gastric fundus and corpus mucosa, in agreement with literature (15). Neither the normal squamous epithelium of the esophagus nor simple reflux esophagitis showed any ghrelin expression. The detection of ghrelin mRNA in the esophagus from histologically uncontrolled specimens might possibly derive from hiatus herniation, gastric heterotopia or Barrett's mucosa, where we also detected some ghrelin (16). In Barrett's mucosa, the aberrant expression of ghrelin has been discussed as a protective adaptation of the mucosa (17).

In the normal corpus mucosa, ghrelin-positive cells were interspersed between parietal cells and chief cells in the lower part of the fundic glands. *H. pylori* gastritis initially affects the upper parts of the fundic glands and represses the number of ghrelin-positive cells to the lower part of the fundic glands. It has

been shown by serological data from *H. pylori* patients that ghrelin values are diminished, and are restored after eradication therapy (18). It is known that atrophic autoimmune gastritis can either occur spontaneously or can be triggered by chronic *H. pylori* infection. In both cases, autoimmune antibodies against parietal cells destroy the fundic glands and promote atrophy. Two kinds of metaplasia occur, *i.e.* intestinal metaplasia and pseudopyloric metaplasia. The latter has been recently called spasmolytic polypeptide-expressing metaplasia (SPEM). Both forms of metaplasia have been found to be associated with gastric cancer (2, 19), and in both kinds of metaplasia, gastro-protective ghrelin-expressing cells are lost, except for their presence in neuroendocrine cell hyperplasia (*Fig. 2*), which will be discussed later. In general, serum ghrelin levels were associated with gastric atrophy and led to the attribution of ghrelin as a bedside biomarker for mucosal integrity (18, 20, 21). The findings in gastric metaplasia of the duodenum were similar to those in the gastric foveolar epithelia and did not show many ghrelin cells.

Ghrelin-producing neuroendocrine cells make up 10–20% of all neuroendocrine cells of the gastric corpus mucosa (5). Double immunofluorescent staining of ghrelin and chromogranin highlighted this composition, and was even stronger in autoimmune gastritis, where ghrelin-positive cells comprised up to 50% of the investigated nodules of neuroendocrine cell hyperplasia (*Fig. 3*), as also reported by others (22). Neoplastic ghrelinomas have also been described (22-25). Taking this into account, the widely used term enterochromaffin-like cell hyperplasia should be revisited as ghrelin-positive cells are affected as well. Ghrelin-expressing cells are not a type of enterochromaffin-like cell, but intermingle with them as derivatives from type A-like cells. Linear and nodular endocrine cell hyperplasia should therefore be preferred as neutral terms. However, the idea of enterochromaffin-like cell hyperplasia is derived from the idea of a direct gastrin influence on these cells (19), which still has to be shown for ghrelin-positive cells. Gastrin is known to have growth stimulating effects on neuroendocrine cells in the gastric fundus (18, 19) but it is unknown if it interacts with ghrelin-expressing cells physiologically. However, we could confirm the existence of the gastrin/cholecystokinin (CCKB) receptor for the first time on human ghrelin-positive cells, which corresponds to data from animal models (9). The finding of this receptor on ghrelin-expressing cells is a prerequisite to a possible influence by gastrin.

Investigations into the direct effects of gastrin on ghrelin-positive cells have provided controversial results (9, 21, 26-28). This might be due to the models used, which mainly are based on serological data or animal models. As we have shown, the gastric hormone system has numerous interactions. Beyond a role of gastrin, other hormones like histamine and somatostatin have been suggested to influence ghrelin (29-32). Thus, gastrin administration to an intact organism might possibly show counter-reactions or impairment, *e.g.* if gastrin knock-out mice are used (33). We chose a primary cell culture model to further avoid systemic reactions and focus on the direct effects of gastrin on the ghrelin mRNA expression profile. Indeed, we observed dose-dependent ghrelin suppression by gastrin (*Fig. 4*).

Transferring this into the observed pathophysiological model of autoimmune gastritis, two effects regarding ghrelin can be postulated. The distribution pattern of ghrelin-positive cells is remodeled with a remarkable decrease in the total number of ghrelin-expressing cells in the atrophic mucosa, but a compensatory increase within neuroendocrine cell hyperplasia. Even if this effect presumably equilibrates the number of ghrelin cells, high levels of gastrin could be regarded as an additional mechanism for the serological suppression of ghrelin in atrophic gastritis (18, 21).

As further evidence, we found a strong inverse correlation between plasma gastrin and ghrelin levels. We compared two different situations of increased gastrin levels. It is known that proton pump inhibition leads to a compensatory increase in gastrin due to the rising pH values in the stomach (18). This short-term effect leads to only a slight and not significant increase of gastrin and therefore does not affect the corresponding ghrelin levels. However, if gastrin levels are constantly elevated, as seen in autoimmune gastritis (27), ghrelin levels are strongly repressed (*Fig. 5*).

Due to processing time, very sensitive active ghrelin levels were not measurable. Our study rather focused on preprandial levels of ghrelin and gastrin. However, both hormones show a circadian rhythm (34), which might be influenced by the direct interaction of both hormones at different plasma levels. Further physiological studies are warranted in this direction.

In conclusion, we investigated the impact of ghrelin in several pathological situations of the upper gastrointestinal tract. Especially in autoimmune gastritis, we were able to find evidence for a new gastrin-mediated mechanism for ghrelin suppression as a gastro-protective marker. In gastric cancer, no ghrelin-expressing cells were visible. Ghrelin might therefore be an additional relevant marker for local homeostasis of the gastric mucosa and might play a role in the gastric carcinogenesis model of gastritis - atrophy/metaplasia - carcinoma sequence (2, 19).

Acknowledgements: The study was supported by grants from the Bavarian Society for Gastroenterology and the Interdisciplinary Centre for Clinical Research (IZKF) Erlangen. We are grateful to Prof. Prinz, 2nd Department of Internal Medicine, Technical University Munich, Germany, who helped us to establish the primary culture techniques. Furthermore, we thank Katja Sesselmann and Angela Neumann for the excellent technical assistance.

Conflict of interests: None declared.

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Received: September 30, 2013
Accepted: December 9, 2013

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