Summary. Ghrelin cell density in the gastrointestinal tract of animal models of human diabetes type 1 and 2 was investigated. The animals used were non-obese diabetic (NOD) mice and obese diabetic mice. Ghrelin cells were detected by immunohistochemistry and quantified by computerized image analysis. Ghrelin-immunoreactive cells were detected in all animals studied. They were abundant in the oxyntic mucosa, patchy and few in the duodenum and rare in the colon. The density of ghrelin-immunoreactive cells decreased in diabetic, pre-diabetic NOD mice and in obese diabetic mice as compared to controls, though not statistically significant. It was concluded that the reduced density of ghrelin-immunoreactive cells in animal models of human diabetes type 1 and 2 might explain the slow gastric emptying and slow intestinal transit found in diabetes gastroenteropathy.

Key words: Ghrelin, Diabetes gastroenteropathy, NOD-mice, Obese-diabetic mice

Introduction

Ghrelin, a 28-amino acid peptide hormone, was discovered in 1999 and was identified as the endogenous ligand for the G-protein-coupled growth hormone secretagogue receptor (GHS-R). It has been found in several different tissues in humans but it is primarily synthesized in specific endocrine cells, designated X/A-like cells, in the gastrointestinal mucosa (Kojima et al., 1999; Date et al., 2000). Ghrelin has been found to serve numerous functions, the best known is its GH-releasing effect in the pituitary, where it acts synergistically with GH-releasing hormone (Kojima et al., 1999; Hataya et al., 2001). Ghrelin also increases appetite and feeding (Wren et al., 2001). It stimulates orexigenic pathways by acting through neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons in the arcuate nucleus of the hypothalamus (Hosoda et al., 2002; Cowley and Grove, 2004). Ghrelin accelerates gastrointestinal motility and increases hydrochloric acid secretion in rodents. The exact mechanism by which gastrointestinal motility is stimulated by ghrelin is not known, but it seems that both local and central pathways are involved (Masuda et al., 2000; Fujino et al., 2003).

Gastrointestinal symptoms such as nausea and vomiting, diarrhoea, constipation, and abdominal pain occur in 25-76% of patients with diabetes mellitus (Feldman and Schiller, 1983; Locke III, 1995; Nilsson, 1996; Schwartz et al., 1996; Spångéus et al., 1999). These symptoms are believed to be caused by gastrointestinal dysmotility (Camilleri, 1996; Koch, 1999). The cause of the gastroenteropathy dysmotility in diabetic patients seems to be multifactorial. Autonomic neuropathy, hyperglycaemia/hyperinsulinaemia and abnormal gut neuroendocrine system are proposed to be the most important factors (Björnsson et al., 1994; Camilleri, 1996; El-Salhy et al., 1998; El-Salhy and Spångéus 1998a,b; Spångéus and El-Salhy, 1998a,b; El-Salhy, 2002, 2005).

As ghrelin seems to play an important role in regulating gastrointestinal motility, the present study was undertaken to establish whether gastrointestinal ghrelin cell density is affected in animal models of human diabetes type 1 and type 2.

Material and methods

Animal models

As a model for human diabetes type 1, female 22-24-week old non-obese diabetic (NOD) mice (Bomholtgård Breeding and Research Centre, Denmark) were used. The characteristics of these animals are given in detail elsewhere (El-Salhy et al., 1998). Shortly, diabetic mice had developed grade 3-4 insulitis and had glucosuria, severe weight loss and significantly reduced pancreatic insulin concentration, and ß-cell density. The pre-diabetic mice also showed severe weight loss, but had no glucosuria. They had grade 1-2 insulitis but...
normal pancreatic insulin concentration and β-cell density. As a control group, age- and sex-matched mice from a non-diabetic sister strain of the NOD-mouse, namely BALB/cJ were used (Bomholtgård). Female 20-week old, obese, homozygote ob/ob mice, were used as a model of human diabetes type 2 (Umeå/Bom-ob; Bomholtgård). Age- and sex- matched, non-diabetic, lean, homozygote null (+/+ ) mice from the same strain were used as controls (Bomholtgård). The animals were kept in cages, 5 in each cage, in artificially illuminated rooms, fed a standard chow (Astra-Ewos AB, Södertälje, Sweden) and given water ad libitum. The number of animals in the groups of the diabetic NOD mice, pre-diabetic NOD mice and controls was 10, 9 and 8 mice, respectively. Ten obese diabetic mice and their controls were included in the study. After overnight fast the animals were killed in a CO₂ chamber and the gastrointestinal tract was dissected out. The corpus part of the stomach, the proximal duodenum and the distal colon were fixed overnight in 4% paraformaldehyde, embedded in wax and cut at 5 µm.

**Immunocytochemistry**

Sections from stomach corpus, proximal duodenum and distal colon from each mouse were immunostained by avidin-biotin complex (ABC) method (Dako Cytomation, Glostrup, Denmark) as described in detail earlier (El-Salhy et al., 1993). Briefly, incubation with the anti-ghrelin antibody (Phoenix Pharmaceuticals, Belmont, CA, USA, code no H03131 dilution 1:1600) was done overnight at room temperature. The incubation with the secondary antibody (biotinylated swine anti-rabbit antibody IgG) was followed by incubation with avidin-biotin-peroxidase complex. Both were diluted at 1:200 and incubation was performed at room temperature for 30 min. The peroxidase was detected by immersing the slides in a solution containing 50 ml Tris-buffer (ph 7.4), 10µl 30% H₂O₂ and 25 mg diaminobenzidine tetrahydrochloride (DAB). The sections were briefly counterstained in haematoxylin. Negative specificity controls were obtained by replacing the primary antibody with 1% bovine albumin, and by pre-incubation of the antibody with excessive antigen (5 mg/ml diluted antibody) for 12 h at 4°C.

**Computerized image analysis**

This was done using Leica’s Quantimet 600MC image processing and analysis system (Leica, Cambridge, England). The program used in this system was QWIN (Windows-based image analysis tool kit, version 2.6). In addition, the system included QUIPS (version 2.6), an interactive programming system. When using x20 objective, each pixel in the computer monitor corresponds to 0.173 µm, and the frame (field) represents areas of 5436 µm². Prior to quantification, the slides were coded and the performer was unaware of the slide identities. Measurements were made in 20 randomly chosen fields for each mouse and gastrointestinal part. These fields were selected from three to four sections, at least 50 mm apart. The number of ghrelin-immunoreactive cells and the area of epithelial cells were determined using an automated standard sequence analysis operation, created by QUIPS, as described earlier in detail (El-Salhy et al., 1997).

**Statistical analysis**

The Kruskal-Wallis non-parametrical ANOVA test and Dunn’s post-tests were used to compare the 3 groups in animal models of human diabetes type 1. The Mann-Whitney non-parametrical test was used for comparison between obese diabetic mice and control group. P-values < 0.05 were considered significant.

**Results**

Ghrelin-immunostained cells were observed in the oxyntic mucosa, in the duodenum and in the colon. They were abundant in the oxyntic mucosa, few in the duodenum and rare in the colon. In the duodenum they

**Fig. 1.** Ghrelin-immunoreactive cells in the oxyntic mucosa of the stomach in a control mouse (A) and in a diabetic NOD mouse.
occurred mostly in the crypts. These cells were round, flask-shaped or triangular (Figs. 1, 2). The few cells encountered in the colon did not allow any reliable quantification. Thus the morphometry was not performed in the colon. Replacing the primary antibody with 1% bovine albumin did not yield any immunostaining. Pre-incubation of the primary antibody with ghrelin abolished completely the immunostaining.

**Oxyntic mucosa of the stomach**

The results of the morphometric measurements in the oxyntic mucosa are illustrated in Fig. 3. The densities of ghrelin-immunoreactive cells were lower in diabetic and pre-diabetic mice than in controls, though this difference was not statistically significant (P=0.4). Similarly, the density of ghrelin-immunoreactive cells in obese diabetic mice was lower than that of controls, though not statistically significant (P=0.16).

**Proximal duodenum**

The density of ghrelin-immunoreactive cells in the duodenum of animal models of human diabetes type 1 and 2 is given in Fig. 4. As in the oxyntic mucosa, the density of ghrelin-immunoreactive cells was reduced in pre-diabetic and diabetic NOD mice as well as in obese diabetic mice, though not statistically significant (P=0.08 and P=0.2, respectively).

**Discussion**

In the present study, ghrelin-immunoreactive cell densities were reduced in the upper gastrointestinal tract.
tracts of animal models of both human diabetes type 1 and 2. Although this difference was not statistically significant, one cannot exclude the possibility of type II error. The present finding of reduced density of ghrelin cells in NOD mice is in agreement with the previously reported results in rats with streptozotocin-induced diabetes (Masaoka et al., 2003). The present observation showed further that this reduction appeared in pre-diabetes state and prior to onset of diabetes. This finding indicates that the reduction in ghrelin-immunoreactive cells is not secondary to the development of the diabetic state.

The obese diabetic mice have a gene mutation resulting in non-functioning leptin (Zhang et al., 1994) with marked hyperphagia and marked obesity (Westman, 1968; Herberg and Coleman, 1977; Ahren and Lundquist, 1982). As ghrelin increases appetite and feeding (Wren et al., 2001), the present finding of reduced ghrelin-immunoreactive cell density in the obese mice with hyperphagia and obesity is rather puzzling.

As ghrelin has been found to accelerate gastrointestinal motility (Masuda et al., 2000; Fujino et al., 2003), the present observation that ghrelin-immunoreactive cell density was reduced in animal models of human diabetes type 1 and 2 might explain the slow gastric emptying and slow intestinal transit found in diabetes gastroenteropathy. Correlation between gastrointestinal motility and ghrelin levels needs, however, to be investigated before any definite conclusion can be drawn.

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References


Ghrelin cells in diabetic mice


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