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Does diabetic state affect co-localization of peptide YY and enteroglucagon in colonic endocrine cells?

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Summary. *Background:* Changes in the numbers of PYY- and enteroglucagon-immunoreactive cells in colon of animal models of human diabetes have been reported. As these peptides co-localize in the same cells it is possible that the observed changes are a result of changes in co-localization. *Methods:* Animal models of human type 1 and type 2 diabetes, namely the non-obese diabetic (NOD) mouse and the obese (ob/ob) mouse, were studied. As controls for the NOD mice, BALB/cJ mice were used and for ob/ob mice, homozygous lean (+/+) mice were used. Tissue samples from colon were double-immunostained for PYY and enteroglucagon according to the indirect immunofluorescence method. *Results:* Co-localization of enteroglucagon and PYY was found in colonic endocrine cells in all groups investigated. Compared with controls, pre-diabetic NOD mice showed a decreased proportion of entero-glucagon/PYY co-localization. There was no difference in diabetic NOD mice or diabetic ob/ob mice when compared with controls. *Conclusions:* Whereas the number of cells containing solely enteroglucagon and solely PYY increases in pre-diabetic NOD mice, production of enteroglucagon in PYY-immunoreactive cells decreases. Although the numbers of PYY and enteroglucagon cells have been reported to be changed in both diabetic NOD mice and in obese mice, the balance between co-expressing and mono-expressing cells seems to be preserved.

Key words: Diabetes, Co-localization, Endocrine cells, PYY, Enteroglucagon, NOD mice, Obese diabetic mice, Colon

Introduction

Peptide YY (PYY) inhibits the secretion of fluid and electrolytes from the intestine (Mannon and Taylor, 1994; Sundler et al., 1989). It is also a potent mediator of ileal brake, inhibits gastric emptying and delays intestinal transit (Mannon and Taylor, 1994; Sundler et al., 1989). Enteroglucagon is a potent incretin substance, i.e. it stimulates the release of insulin from the pancreatic islets (Walsh, 1994). In man, as in other mammals, numerous enteroglucagon- and peptide YY (PYY)-containing endocrine cells are present in the mucosa of the terminal ileum and colon (El-Salhy et al., 1983a,b; El-Salhy and Grimelius, 1983; El-Salhy and Lundqvist, 1984; Sundler et al., 1989; Walsh, 1994). PYY and enteroglucagon co-localize in intestinal L-cells of rat, pig and man (Mannon and Taylor, 1994). PYY also occurs in another cell type, namely intestinal H-cells (El-Salhy et al., 1983a; Mannon and Taylor, 1994). Since PYY and enteroglucagon are synthetic products of distinct precursors, it has been suggested that the L-cell expresses two different genes (Mannon and Taylor, 1994).

Patients with diabetes mellitus often suffer from disturbed motility in the gut (Feldman and Schiller, 1983; Valdovinos et al., 1993; Locke, 1995), and it may be that these peptides are involved in the pathogenesis of this disturbance. In support of this assumption, recent findings in an animal model of human type 1 diabetes, namely the non-obese diabetic (NOD) mouse, showed an increase in the number of enteroglucagon-immuno-reactive (IR) cells in pre-diabetic NOD mice and a decrease in the number of PYY-IR cells in diabetic animals (Spångéus and El-Salhy, 1998). Furthermore, both PYY- and enteroglucagon-IR cells have been found to decrease in number in the obese diabetic mouse, an animal model of human type 2 diabetes (Spångéus and El-Salhy, 1999). It is not clear, however, whether the change in the numbers of PYY- and enteroglucagon-IR cells in these animal models is a real change, or is caused by the production of only one peptide instead of two in these cells.

The present study was undertaken to establish whether the diabetic state affects the co-localization of PYY and enteroglucagon. For this purpose, the co-localization of PYY and enteroglucagon was investigated in the colon of animal models of human type 1 (Makino et al., 1980; Kolb, 1987; Tochino, 1987) and type 2 (Herberg and Coleman, 1977; Lord and Atkins, 1985) diabetes; namely the NOD and ob/ob diabetic mice.

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Materials and methods

Animals

Female 22-24-week-old non-obese diabetic NOD/Bom mice (Bomholtgård Breeding and Research Centre, Denmark) were studied. For control purposes, age and sex matched BALB/cJ mice (Bomholtgård), which is a sister strain to NOD mice (Fujishima et al., 1989), were used. The NOD mice were divided into pre-diabetic and diabetic animals according to criteria described in detail elsewhere (El-Salhy et al., 1998). Thus, the pre-diabetic NOD mice were not glucosuric, had a reduced body weight, normal pancreatic insulin content, decreased (though not statistically significantly so) pancreatic islets and insulin cell volume density, and had a 1-2 grade insulitis. Diabetic NOD mice had glucosuria, significantly reduced body weight, significantly low pancreatic insulin content, significantly low volume density of pancreatic islets and insulin cells, and a 3-4 grade insulitis.

Homozygous obese (ob/ob) male mice (Umeå/Bom-ob, Bomholtgård), 20 weeks old, were also studied. As controls, non-diabetic age and sex matched homozygous lean (+/+) mice (Bomholtgård) were used. The data on these animals, as provided by the supplier, were as follows: body weight of the diabetic mice: 101.5±2.7 g; plasma glucose level: 11.6±0.3 mmol/l; plasma insulin: 24.0±7.4 ng/ml; all values expressed as mean+SEM. The corresponding values for lean controls were 42.2±0.6 g, 7.4±0.4 mmol/l and 2.3±0.5 ng/ml.

Each group comprised 7 animals. To adapt them prior to being killed, the mice were kept for one week in our vivarium, 5 in each cage, in a room with 12 h light/dark cycle. They were given standard pellet diet (Astra-Ewos AB, Södertälje, Sweden) and water ad libitum. After an overnight fast, the mice were killed in a CO_2 chamber and the distal colon was excised. The local committee on animal ethics at Umeå University approved the investigation.

Immunocytochemistry

The tissue specimens were fixed overnight in 4% buffered formaldehyde, embedded in paraffin wax and cut into 5 μm-thick sections. The sections were double-immunostained for PYY and enteroglucagon, using the indirect immunofluorescence method as described earlier (Lewis et al., 1993), with slight modifications. Briefly, the hydrated sections were rinsed for 20 min in a 1% solution of Triton X-100 (Kebo Lab, Sweden) in 0.01M phosphate-buffered saline (PBS) pH 7.2, containing 0.1 sodium azide as preservative, followed by incubation for 15 min in 5% normal swine serum. They were then incubated for 1 h at 37 °C with one of the primary antisera diluted in PBS. After incubation with this antiserum, washes in PBS and incubation for another 15 min in 5% normal swine serum, the sections were immersed in tetramethylrhodamine isothiocyanate (TRITC)-conjugated swine anti-rabbit IgG (diluted 1:40; Dakopatts, Glostrup, Denmark) for 30 min at 37 °C. Before applying the other primary antiserum, the sections were treated with normal rabbit serum for 15 min, followed by incubation for 60 min at 37 °C with F(Ab´)2 fragment (diluted 1:20, Dakopatts). After the PBS washes and 15 min treatment with 5% swine serum, the second primary antiserum was applied to the sections for 60 min at 37 °C. Following PBS washes and treatment with normal swine serum, fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG (diluted 1:40, Dakopatts) was applied to the sections for 30 min at 37 °C. The slides were then mounted in glycerol:PBS (1:1) and examined in a Leitz Orthoplan photomicroscope equipped with epifluorescence optics.

The primary antibodies used were rabbit anti-glucagon (diluted 1:100, code no. R841303-B4, cross-reacted with both pancreatic glucagon and gut enteroglucagon; Eurodiagnostica, Malmö, Sweden) and rabbit anti-PYY (diluted 1:100, code no. R781101-B37-1, Eurodiagnostica).

Specificity controls were performed as described previously (Beesley, 1993; El-Salhy et al., 1993). Briefly, they included replacement of the primary antibody by non- immune rabbit serum as well as pre-incubation of the primary antibody, for 24 h at 4 °C, with the corresponding or a structurally related peptide. Thus, anti-PYY was incubated with pancreatic polypeptide (PP), neuropeptide Y (NPY), and anti-glucagon with pancreatic glucagon, secretin, vasoactive intestinal peptide (VIP), and gastric inhibitory peptide (GIP). Positive controls were obtained by including sections from human colon. The controls also included reversal of the order of antigen detection.

Analysis

In each mouse, 30 PYY- and 30 enteroglucagon-IR cells from two different sections, at least 50 μm apart, were examined. The presence of enteroglucagon in PYY-IR cells and PYY in enteroglucagon-IR cells was recorded. The examination was performed with a x40 objective.

Statistical Analysis

Comparisons between the two groups were performed using the non-parametric, Wilcoxon (Mann-Whitney) test. P-values below 0.05 were considered significant.

Results

Immunocytochemistry

Co-localization of enteroglucagon and PYY in colonic endocrine cells was found in all mouse groups investigated. In a number of cells, however, either PYY or enteroglucagon immunoreactivity was found (Fig. 1).

The numbers of these different types of cells varied in different mouse groups (Fig. 2). Thus, in pre-diabetic NOD mice there was a decreased proportion of enteroglucagon-IR cells showing PYY immunoreaction, as well as a decreased proportion of PYY-IR cells showing enteroglucagon immunoreaction, when compared with controls. No difference was found in diabetic NOD mice or diabetic ob/ob mice as compared to controls.

Specificity controls

Pre-incubation of anti-PYY with PYY, and anti-glucagon with pancreatic glucagon, as well as replacing the primary antisera with non-immune rabbit serum resulted in no immunostaining. Pre-incubation of anti-PYY with PP and NPY, and anti-glucagon with secretin, VIP and GIP, did not affect the staining. Both anti-PYY and anti-glucagon produced immunostaining of cells in human colon. Reversal of the order of antigen detection had no effect on the outcome of the immunostaining.

Discussion

The present study has shown that PYY and enteroglucagon co-localize in murine colonic endocrine cells, which is similar to earlier reports in rat, pig and man (Mannon and Taylor, 1994). It has further shown

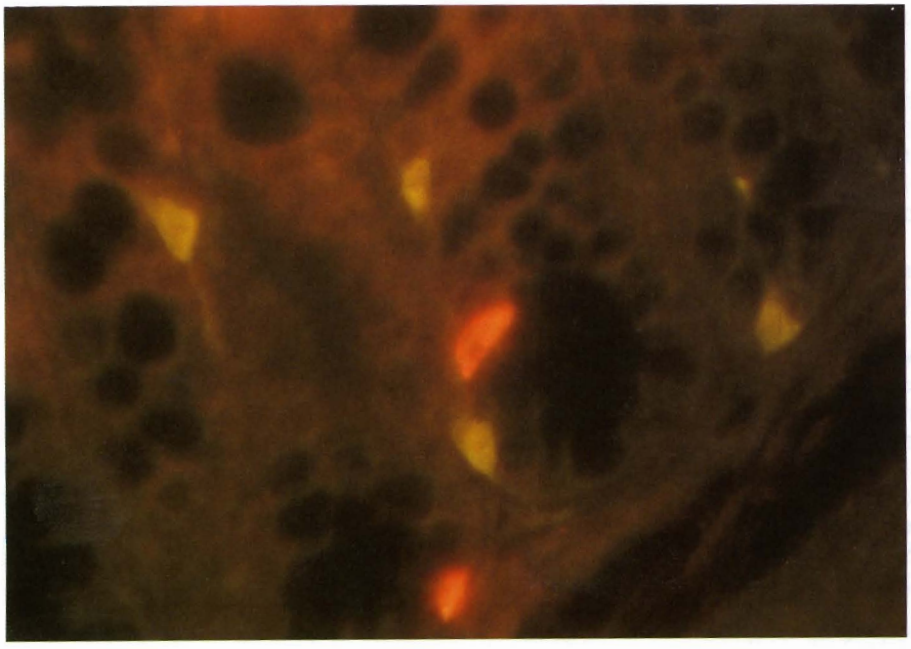

Fig. 1. PYY-(green) and enteroglucagon-immunoreactive cells (red). x 350

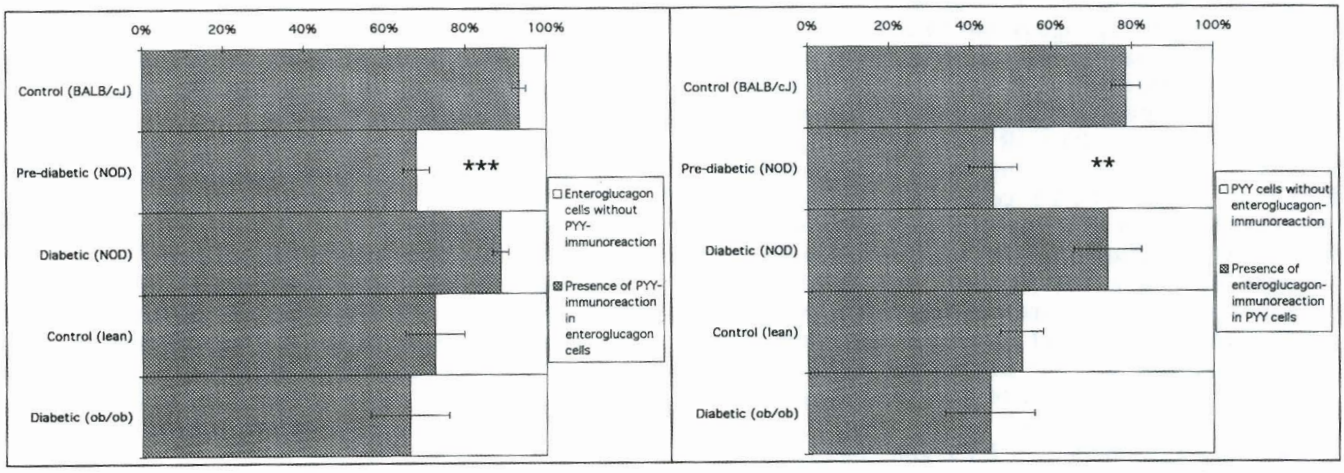

Fig. 2. Proportion of enteroglucagon cells showing presence of PYY immunoreaction **(A)**, and proportion of PYY cells showing presence of enteroglucagon immunoreaction **(B)** in NOD mice, obese (ob/ob) mice and control mice (mean±SEM). *: P<0.05; **: P<0.01; ***: P<0.001.

that cells showing either a PYY- or an enteroglucagon-immunoreaction also occur. Whereas most of the murine colonic enteroglucagon-IR cells expressed PYY, a slightly smaller proportion of PYY-IR cells expressed enteroglucagon. This is consistent with an earlier observation that enteroglucagon-IR cells are less numerous than PYY-IR cells in the mouse colon (Spångéus and El-Salhy, 1998, 1999; Sandström et al., 1998). The degree of PYY and enteroglucagon co-localization, however, differed in the different mice strains studied.

The diabetic state appears not to affect the degree of co-localization of PYY and enteroglucagon in murine colonic cells, indicating that this state does not affect the peptide processing in these cells. This is so regardless of whether the diabetic state arises because of insulin deficiency (as in NOD mice), or because of peripheral resistance to insulin (as in obese diabetic mice). In diabetic NOD mice, the PYY-IR cells have previously been found to decrease in number, whereas the number of enteroglucagon-IR cells did not differ from that of controls (Spångéus and El-Salhy, 1998). As the present investigation showed that there was no difference between diabetic NOD mice and controls regarding the proportion of cells displaying both PYY and enteroglucagon immunoreaction, the decrease in the number of PYY-IR cells in diabetic NOD mice observed earlier (Spångéus and El-Salhy, 1998) seems to occur in both co-localized cells and cells containing solely PYY. In obese diabetic mice, PYY-IR and enteroglucagon-IR cells have been found to decrease in number (Spångéus and El-Salhy, 1999). As in diabetic NOD mice, the proportions of cells containing both PYY and enteroglucagon immunoreaction did not differ from controls, which suggests that the previously reported decrease in number of these cells (Spångéus and El-Salhy, 1999) occurred in both mono- and co-expressing PYY and enteroglucagon cells.

It has been reported earlier that whereas the number of enteroglucagon-IR cells was increased in pre-diabetic NOD mice, the number of PYY-IR cells did not differ from the controls (Spångéus and El-Salhy, 1998). The present finding that pre-diabetic NOD mice showed a decreased proportion of PYY/enteroglucagon co-localization, suggests that the production of enteroglucagon ceased in PYY-IR cells. Consequently the proportion of cells containing solely PYY increased, though the number of PYY cells remained unchanged. The possibility of a disturbance of peptide synthesis at the mRNA level cannot be ruled out. Further studies are required to settle this matter. The increase in enteroglucagon-IR cell count observed earlier (Spångéus and El-Salhy, 1998) might have been the result of an increased number of cells containing solely enteroglucagon, as the proportion of these was significantly increased in the present study.

Although the numbers of PYY and enteroglucagon cells were reported to be changed in the diabetic state, the present study showed that this state had no effect on the co-localization of these peptides in colonic endocrine cells.

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