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Effect of diabetic state on co-localization of substance P and serotonin in the gut in animal models

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Summary. Changes in the numbers of serotonin- and substance P-immunoreactive (IR) cells occur in several animal models of diabetes. It is not known, however, whether these changes are a result of actual cell loss or are caused by modified gene expression in cells showing co-localization of serotonin and substance P. The pattern of mono- and co-expression of serotonin, as well as of substance P, was therefore investigated in gastrointestinal endocrine cells from animal models of human type 1 and type 2 diabetes, namely non-obese diabetic (NOD) and obese diabetic (ob/ob) mice. Immunocytochemical staining by the avidin-biotin complex method was performed for computerized image analysis of each cell type, and by immunofluorescence double staining to study co-localization. Tissues from antrum, proximal duodenum and distal colon were investigated. Co-localization of serotonin- and substance P-IR was found in all investigated parts of the gut. In antrum, substance P immunoreactivity was found exclusively in serotonin-IR cells. In both NOD and ob/ob mice there was a reduced number of substance P-IR cells, but an unchanged serotonin-IR cell count, which thus tallies with a shut-off of substance P expression in antral enterochromaffin cells. In duodenum, both diabetes models showed a decreased number of serotonin-IR cells. Furthermore there was a decreased number of substance P-IR cells in the type 2 model. The proportion of serotonin-IR cells showing substance P-immuno-reactivity was decreased in both diabetic models, thus indicating a shut-off of substance P-gene expression. However, this does not fully explain the changes in duodenum, but the diabetic state probably affects the number of mono-expressed cells as well. In colon, no change was found in diabetic mice regarding co-localization of substance P and serotonin. However, pre-diabetic NOD mice showed a decreased proportion of substance P in serotonin-IR cells, which might be explained by the increased number of serotonin-IR cells, combined with an unchanged number of substance P-IR cells. In conclusion, diabetic animal models of both type 1 and type 2 appear to have a combination of decreased expression of substance P in serotonin-IR cells of both antrum and duodenum, as well as a change in the number of mono-expressed cells. The pattern in colon, on the other hand, seems to be unaffected.

Key words: Diabetes, Endocrine cells, Gastrointestinal, Serotonin, Substance P

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Introduction

Motility dysfunction is common in the gut of diabetic patients (Feldman and Schiller, 1983; Locke, 1995). Both substance P and serotonin play an important role in the regulation of gut motility. Thus, substance P stimulates peristalsis and accelerates gastrointestinal transit, while serotonin stimulates pyloric contraction, small intestinal and colonic motility, as well as accelerating transit through the small and large intestine (Lidberg, 1985; Tally, 1992; Oosterbosch et al., 1993; Goarard et al., 1994; Dockary, 1994; von der Ohe et al., 1994). It is conceivable that a disturbance in these regulatory substances occurs in the gut of diabetic patients. This assumption is supported by recent findings in animal models of type 1 and type 2 diabetes, where abnormalities have been reported concerning substance P and serotonin cells (Goes et al., 1990; Pinto et al., 1995; El-Salhy and Spångéus, 1998a,b; El-Salhy et al., 1998; Spångéus and El-Salhy, 1998, 1999). Substance P and serotonin are both present in endocrine cells in gut mucosa. In humans and most other mammals, substance P and serotonin have been found co-localized in a subtype of the enterochromaffin cell (Dockary, 1994). Co-localization of substance P with serotonin in enterochromaffin cells has not, to the best of our knowledge, been investigated in murine gut.

Previous studies on serotonin and substance P in the gut of animal models of diabetes have been focused on each of these peptide/monoamine separately. It is interesting, however, to ascertain whether the

abnormalities in cell numbers observed earlier (Goes et al., 1990; Pinto et al., 1995; El-Salhy and Spångéus, 1998a,b; El-Salhy et al., 1998; Spångéus and El-Salhy, 1998, 1999) were a result of changes in absolute cell numbers, or whether they resulted from switching on/off of gene expression in the co-localized cells.

The non-obese diabetic (NOD) mouse is a strain genetically predisposed to develop a condition similar to human type 1 diabetes, characterized by an abrupt onset of glucosuria, hypoinsulinaemia, polyphagia, polydipsia, polyuria, hypercholesterolaemia and ketonaemia (Makino et al., 1980; Kolb, 1987; Tochino, 1987). Mice homozygous of the autosomal-recessive *ob*-gene (ob/ob-mice) are characterized by hyperinsulinaemia, insulin-resistant hyperglycaemia, and obesity (Herberg and Coleman, 1977; Lord and Atkins, 1985). These metabolic disturbances resemble those found in human type 2 diabetes.

The aims of this study were to investigate the co-localization of serotonin and substance P in mice, and to investigate whether the diabetic state influences peptide/monoamine expression in the cells in which these substances are co-localized, or influences the absolute number of any of these endocrine cells.

Material and methods

Animals

As an animal model for human type 1 diabetes, 22-24-week-old female pre-diabetic and diabetic non-obese diabetic (NOD/Bom) mice (Bomholtgård Breeding and Research Centre, Denmark) were used. These animals have been characterized in detail elsewhere (El-Salhy et al., 1998). In brief, the pre-diabetic NOD mice did not have glucosuria, but had reduced body weight, a 1-2 grade insulitis, normal pancreatic insulin content and reduced (but not statistically significant) pancreatic islet and insulin cell volume density. Diabetic NOD mice had glucosuria, significantly reduced body weight, a 3-4 grade insulitis, significantly low pancreatic insulin content and significantly low volume density of pancreatic islets and insulin cells. Age-matched female mice of the sister strain BALB/cJ (Bomholtgård) served as controls. Homozygous male obese diabetic (ob/ob) mice (Umeå/Bom-ob, Bomholtgård), 20 weeks old, were used as an animal model for human type 2 diabetes. As controls, non-diabetic age-matched male homozygous lean (+/+) mice (Bomholtgård) were used. According to the supplier the diabetic obese mice had a body weight of 101.5±2.7 g, their plasma glucose level was 11.6±0.3 mmol/l and plasma insulin, 24.0±7.4 ng/ml (the 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 allow them to adapt to the vivarium conditions, they were kept there for one week, until sacrifice. They were housed 5 to each cage in a room with a 12 h light/dark cycle and were given standard pellet diet (Astra-Ewos AB, Södertälje, Sweden) and water *ad libitum*. After an overnight starvation, the mice were killed in a CO_2 chamber.

The antrum, proximal duodenum and distal colon were excised, and were fixed overnight in 4% buffered formaldehyde. The local committee on animal ethics at Umeå University approved the investigation.

Microwave antigen retrieval

The sections were treated as described in detail elsewhere (Nyhlin et al., 1997). Briefly, they were hydrated, and immersed in 0.01M citrate buffer, pH 6, in plastic Coplin jars, which were placed in a microwave oven for three boiling cycles of 5 min each, at maximum power (650 W). For each run, the sections were quickly transferred to a new jar with fresh retrieval solution. After the last run, the slides were allowed to cool to room temperature for 20 min, rinsed in Tris-buffer, pH 7.6, and then immunostained.

Immunocytochemistry

The tissue specimens were embedded in paraffin wax and sectioned at 10 μm thick. The sections were then immunostained with or without antigen retrieval with the avidin-biotin complex (ABC) method (Dakopatts, Glostrup, Denmark) as described in detail earlier (El-Salhy et al., 1993). In brief, endogenous peroxidase was blocked by incubation in 0.5% H_2O_2 for 30 min. The slides were then treated with 1% bovine serum albumin for 10 minutes to occupy unspecific binding sites, followed by overnight incubation with the primary antiserum in a moisture chamber. Biotinylated swine anti-rabbit IgG (dilution 1:100) was applied for 30 min, followed by avidin-biotin complex for 30 min. The sections were rinsed in 50 ml Tris-buffer containing 10 μl of 30% H_2O_2 and 25 mg diaminobenzidine tetrahydrochloride (DAB) and counterstained lightly with Mayer's haemotoxylin. All steps were performed at room temperature. The primary antisera used were rabbit anti-substance P (diluted 1:1000, code no. SP2-840530 B45-1, Eurodiagnostica, Malmö, Sweden), rabbit anti-substance P (diluted 1:1000, code no. B069082, BioGenex, San Ramon, California, USA) and anti-serotonin (diluted 1:400, code no. R 871204 B56-1, Eurodiagnostica).

Double immunostaining was performed by a procedure using indirect immunofluorescence (Lewis-Carl et al., 1993). Briefly, the 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, followed by incubation for 15 min in 5% non-immune swine serum. They were then incubated with one of the primary antisera, diluted in PBS, for 1 h at 37 °C. After washes in PBS and incubation for another 15 min in 5% normal swine serum, the sections were immersed in tetramethyl-rhodamine isothiocyanate (TRITC)-conjugated swine anti-rabbit IgG (diluted 1:40, Dakopatts, Glostrup, Denmark) for 30 min at 37 °C.

This was followed by treatment with normal rabbit serum for 15 min and by incubation with goat anti-rabbit $F(Ab')_2$ fragment (diluted 1:20, Dakopatts) for 60 min at 37 °C. After PBS washes and a 15-min treatment with 5% swine serum, the second primary antiserum was applied to the sections for 60 min at 37 °C. Following the PBS washes and normal swine serum treatment, 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-substance P (diluted 1:100, code no. B069082, BioGenex) and rabbit anti-serotonin (diluted 1:100, code no. NT102, ETI, New Jersey, USA).

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 anti-substance P with 50 μg synthetic substance P, and serotonin, for 24 h at 4 °C as well as anti-serotonin with 50 μg of serotonin or substance P. Positive controls were obtained by including sections from human colon in the immunostaining. The controls also included reversal of the order of antigen detection.

Computerized image analysis

Quantification of immunoreactive cells was performed as described earlier (El-Salhy et al., 1997). In brief, an Olympus microscope (type BX50) was linked to the Quantimet 500MC image processing and analysis system (Leica, Cambridge, England). The software in this system comprised; QWIN (version 1.02), an image analysis program and QUIPS (version 1.02), an interactive programming system. The quantification was performed in an automated standard analysis sequence as described earlier (El-Salhy et al., 1997). In brief, the number of cells was counted using field measurements, and the area of epithelium by threshold settings. In each mouse and for substance P and serotonin, twenty fields were randomly chosen (from 2-4 different sections, separated from each other by 100 μm) and were investigated in a x20 objective. At this magnification, each pixel of the computer image corresponded to 0.414 μm and each field seen in the monitor represented an area of 0.04 mm^2 of the tissue.

Co-localization

In each mouse 30 substance P cells from two different sections (separated by at least 100 μm) were analysed regarding their serotonin content. In addition, 30 serotonin cells were analysed regarding their substance P content. The analyses were performed with a Leitz Orthoplan Photomicroscope equipped with epifluorescence optics, using a x40 objective.

Statistical analysis

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

Results

Immunocytochemistry

Substance P-IR cells were clearly distinguished in sections immunostained after microwave antigen retrieval, but not in sections immunostained without such treatment. On the other hand, substance P-IR nerve fibres were observed in sections both with and without microwave antigen retrieval treatment. The subsequent description and morphometric analysis were done on sections immunostained following microwave antigen retrieval. Substance P immunoreactivity was found in nerve fibres and neurones of the myenteric and submucosal plexi as well as in endocrine cells in the mucosa of the antrum, duodenum, and colon of NOD mice, ob/ob-mice and control mice. The substance P-IR endocrine cells were numerous in duodenum and colon, but sparse in antrum. Serotonin-IR endocrine cells were numerous in the mucosa of all investigated parts of the gut. Both substance P- and serotonin-IR endocrine cells varied in shape, from round, flask-shaped, to triangular, often with a basal process.

Specificity controls, including replacement of primary antisera with non-immune rabbit serum, as well as pre-incubating the primary antisera with the corresponding antigen, i.e. substance P or serotonin, resulted in no immunostaining. Antibodies to both substance P and serotonin immunostained cells in human gut.

Computerized image analysis

The numbers of both serotonin-IR and substance P-IR cells are presented in Figs. 1, 2. In antrum, the number of substance P-IR cells was decreased in pre-diabetic and diabetic NOD mice as well as in diabetic ob/ob mice, compared with respective control mice. No difference was found in the number of serotonin-IR cells in either NOD or ob/ob mice vs respective controls.

In duodenum, whereas the number of substance P-IR cells decreased in diabetic ob/ob mice vis-à-vis their lean controls, no difference was found between the NOD mice and their controls. The number of serotonin-IR cells was significantly decreased in both pre-diabetic and diabetic NOD mice as well as in diabetic ob/ob mice, vis-à-vis their respective controls.

In colon, no statistical difference was found between NOD mice or ob/ob mice and their respective controls regarding numbers of substance P-IR cells. Whereas serotonin-IR cells were more numerous in pre-diabetic NOD mice than in controls, no difference was found in diabetic NOD mice. In ob/ob mice, the number of

serotonin was decreased vs control mice.

Co-localization

Co-localization of substance P and serotonin was found in all investigated parts of the gut. In antrum, all of the few existing substance P cells seemed to coexist with the serotonin, which is why no further quantification was made. In duodenum and colon there were pure serotonin cells, pure substance P cells, as well as co-localized serotonin/substance P cells (Fig. 3).

Co-localization of serotonin and substance P is presented in Fig. 4. In diabetic NOD mice as well as in diabetic ob/ob mice, the proportion of duodenal serotonin-IR cells showing substance P-immunoreactivity was reduced vis-à-vis their controls. No statistical difference was found in pre-diabetic mice *vs* controls regarding the proportion of duodenal serotonin-IR cells containing substance P. In colon, pre-diabetic NOD mice had a decreased proportion of serotonin-IR cells showing substance P-immunoreactivity, as compared with controls. No similar difference was found for either diabetic NOD mice or diabetic ob/ob mice vis-à-vis their respective controls.

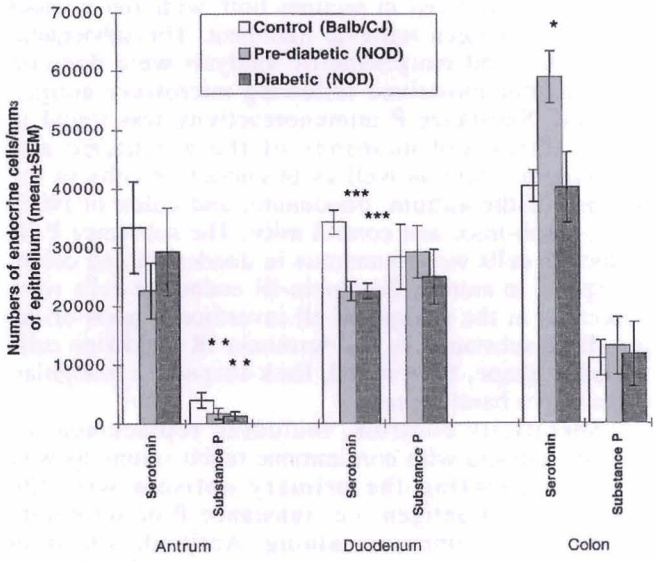

Fig. 1. Numbers of serotonin- and substance P-IR endocrine cells in the gut of NOD mice and control mice (mean±SEM). *: P<0.05; **: P<0.01; ***: P<0.001.

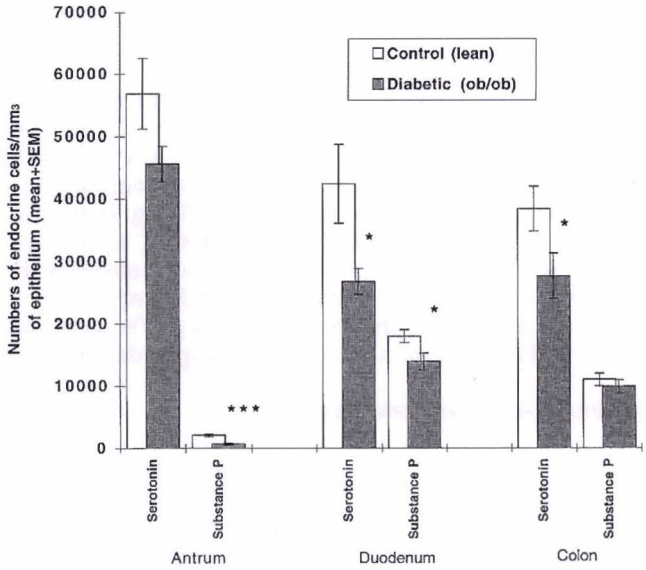

Fig. 2. Numbers of serotonin- and substance P-IR endocrine cells in the gut of obese (ob/ob) mice and control mice (mean±SEM). *: P<0.05; **: P<0.01; ***: P<0.001.

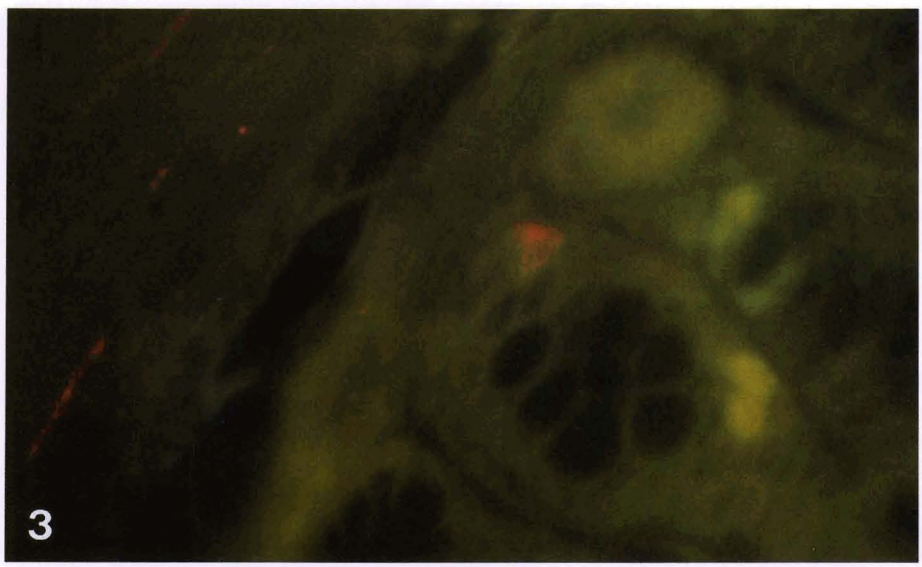

Fig. 3. Mono-expressed substance P cells (red) and serotonin cells (green) as well as co-localized substance P/serotonin cells (orange). x 350

Discussion

The present study showed a high degree of co-localization of substance P and serotonin in endocrine cells of the mouse gut. It is worth noting that substance P-IR cells could be detected only after microwave antigen retrieval. The way in which antigen retrieval affects tissues is not yet known. It had been assumed, however, that it acts by breaking the formaldehyde-induced links between epitopes and unrelated proteins, by extraction of diffusible blocking proteins and probably by precipitation of proteins and rehydration of the tissue section thus allowing better penetration of the antibody (Suurmeijer and Boon, 1993). In any way this pre-treatment of the sections appears to unmask epitopes for substance P, allowing its detection. In mice, most of enterochromaffin cells appeared to express substance P and serotonin in all investigated segments of the gastrointestinal tract. There appears to be a species difference in the distribution of mucosal endocrine cells containing substance P. Thus, substance P-IR cells have been found in the stomach and intestine of marmoset, and in the intestine of dog and man, but are absent in rat and guinea pig (Dockary, 1994). It is worth noting, however, that those results in these species were obtained without microwave antigen retrieval and that application of this pretreatment may give different results.

In the present study both substance P- and serotonin-IR cells were found to be affected by the diabetic state. This observation agrees with previous findings in models of diabetes type 1 and type 2 (Goes et al., 1990; Pinto et al., 1995; El-Salhy and Spångéus, 1998a,b; El-Salhy et al., 1998; Spångéus and El-Salhy, 1998). All differences, except for serotonin in pre-diabetic colon, were decreases in the cell count of serotonin and/or substance P. These changes in serotonin- and substance P-producing endocrine cells in animal models of diabetes emphasize the possibility that these signal substances might be involved in the gut dysmotility observed in diabetic animals (Diani et al., 1979; Scott and Ellis, 1980) and might be of some importance regarding the motility disorders seen in diabetic patients.

As regards co-localization, substance P-immunoreactivity was found exclusively together with serotonin-immunoreactivity in antrum of all mouse groups. Thus, the finding of reduced numbers of substance P-containing cells, together with unchanged serotonin in NOD and ob/ob mice, would seem to result from a decrease in substance P gene expression in serotonin cells. In duodenum also there was a lower proportion of serotonin-IR cells expressing substance P, thus indicating a gene expression shut off. However, this cannot fully explain the changes reported in absolute numbers of substance P-IR and serotonin-IR cells (Figs. 1 and 2) in duodenum. Thus, it is conceivable that diabetic mice, in addition, underwent changes in the numbers of mono-expressed cells.

In colon, no change was found in diabetic mice regarding co-localization of substance P and serotonin. However, pre-diabetic NOD mice showed a reduced proportion of substance P in serotonin-IR cells, which is probably explained by the increased number of serotonin-IR cells in combination with an unchanged number of substance P-IR cells. A previous study of colonic enteroglucagon- and peptide YY-IR endocrine cells have similarly reported a preserved pattern of co-localization in diabetic NOD and ob/ob mice, but an altered pattern in pre-diabetic NOD mice (Spångéus et al., 2000).

In conclusion, diabetic mice appear to have reduced expression of substance P in serotonin-IR cells of both antrum and duodenum, while in duodenum the number of mono-expressed cells also appears to be affected. In colon, no change was found in the pattern of co-localization of substance P and serotonin in diabetic mice.

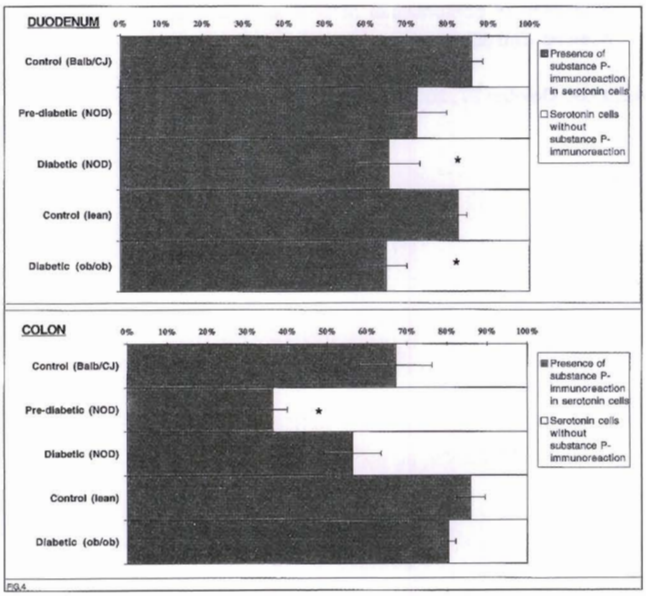

Fig. 4. Proportions of serotonin-IR cells showing presence of substance P-immunoreaction in NOD mice, obese (ob/ob) mice and control mice (mean±SEM). *: P<0.05; **: P<0.01; ***: P<0.001.

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