Myenteric plexus of obese diabetic mice (an animal model of human type 2 diabetes)

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Summary. The myenteric plexus of the gastrointestinal tract was investigated in the obese diabetic mouse, an animal model of human type 2 diabetes. Sections were immunostained by the avidin-biotin complex method, using a general nerve marker, protein gene product 9.5 (PGP 9.5), as well as antibodies to several important neurotransmitters. Computerized image analysis was used for quantification. When diabetic mice were compared with controls, no difference was found in the density of PGP 9.5-immunoreactive (IR) nerve fibres in antrum, duodenum or colon. In antrum and duodenum, diabetic mice showed a decreased number of vasoactive intestinal peptide (VIP)-IR neurons in myenteric ganglia as well a decreased relative volume density in myenteric plexus (though not significantly in antrum, p=0.073). No difference was found regarding VIP-IR nerves in colon. The volume density of nitric oxide synthase (NOS)-IR nerve fibres was decreased in antrum and duodenum of diabetic mice, whereas no difference was found in colon. The density of galanin-IR nerve fibres was decreased in duodenum. Whereas neuropeptide Y (NPY)- and vesicular acetylcholine transporter (VACHT)-IR nerve fibres was increased in density in colon of diabetic mice, no difference was found in antrum and duodenum. Regarding substance P, there was no difference between diabetic and control mice in antrum, duodenum or colon. The present study shows that gut innervation is affected in this animal model of type 2 diabetes, while no difference was found in controls. Antrum, duodenum and colon were studied as to neurotransmitters and the gastrointestinal dysfunction is not established.

Introduction

Patients with type 1 or type 2 diabetes often show complications in the gastrointestinal tract, such as gastroparesis, constipation, diarrhoea and faecal incontinence (Bargen et al., 1936; Kassander, 1958; Feldman and Schiller, 1983; Locke III, 1995). As extrinsic and intrinsic nerves of the gastrointestinal tract are important regulators of gut functions, such as motility, secretion and absorption (Allescher et al., 1991; Ekbland et al., 1991; Rangachari, 1991; Costa and Brookes, 1994), it is possible that changes in these may occur and be involved in the pathogenesis of the gastrointestinal symptoms observed in diabetes patients. To study gut innervation, however, transmural biopsies are needed, which are difficult to obtain from diabetes patients. Thus, animal models of diabetes are often used to study possible disturbances in the diabetic state. In both genetically- and chemically-induced animal models of diabetes type 1, several disturbances in the enteric innervation of the gut have been reported (Ballman and Conlon, 1985; Belai et al., 1985, 1991; Service et al., 1985; Loesch et al., 1986; Dj Giulio et al., 1989; Wrzos et al., 1997; El-Salhy and Spångéus, 1998a; Spångéus and El-Salhy, 1998a; Spångéus et al., 2000). In contrast, there are only a few published studies on the enteric innervation in animal models of diabetes type 2 (Baily et al., 1986; El-Salhy, 1998). The relation of changes in neurotransmitters and the gastrointestinal dysfunction is not established.

The present study was undertaken to investigate the myenteric plexus in different segments of the gastrointestinal tract of a genetically prone, type 2 diabetes animal model, namely the ob/ob-mouse (Herberg and Coleman, 1977; Lord and Atkins, 1985). Thus antrum, duodenum and colon were studied as to represent stomach, small and large intestine. In this study the myenteric plexus was investigated with a general nerve marker, namely protein gene product 9.5, PGP 9.5 (Krammer et al., 1993, 1994), as well as for several important neurotransmitters of the gut, known to regulate motility and secretion.

Material and methods

Animals

Seven 20-week-old, male homozygous obese (ob/ob) diabetic mice (Umeå/Bom-ob, Bomholtgård Breeding
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and Research Centre, Ry, Denmark) were investigated. They were compared with 7 non-diabetic age-matched male homozygous lean (+/+) control mice (Bomholtgård). The animals were kept 5 to each cage in an artificially lit room (12h/12h, light/dark) and were fed standard pellet diet (Astra-Ewos AB, Södertälje, Sweden) and water ad libitum. They were housed in our vivarium for one week to adapt them to the new conditions prior to killing. After overnight starvation, the animals were killed in a CO2 chamber and about 1 cm long specimens of the antrum, proximal duodenum and distal colon were excised. Data on the animals were obtained from the supplier: for the diabetic mice, body weight was 101.5±2.7 g, plasma glucose 11.6±0.3 mmol/liter and plasma insulin 24.0±7.4 ng/ml (mean ± SEM). The corresponding figures for the lean control mice were 42.2±0.6 g, 7.4±0.4 mmol/liter and 2.3±0.5 ng/ml. The local committee on animal ethics at Umeå University approved the investigation.

**Immunocytochemistry**

Tissues were fixed overnight, without applying any tension, in 4% buffered formaldehyde. They were embedded in paraffin wax and cut into 10 μm-thick transversal sections (i.e. perpendicular to the longitudinal muscle layer). After pre-treatment with microwave antigen retrieval to unmask epitopes, the sections were treated in a microwave oven (650 W) for three cycles, each cycle in fresh citrate buffer (pH 6.0). Endogenous peroxidase was inhibited by treatment with 0.5 ml H2O2 (30%) in 50 ml Tris buffer for 10 min, and non-specific binding sites were blocked with 1% bovine serum albumin for 10 min. The primary antibody with non-immune rabbit serum as well as after pre-incubating the primary antibody with corresponding or structurally related peptides. Thus NPY antibodies were pre-incubated with PYY and PP, VIP antibodies with GIP, glucagon, secretin, PHI and CCK, substance P antibodies with neurokinin A, and galanin antibodies with CCK-39, VIP and secretin.

**Computerized image analysis**

Immunoreactive nerves were quantified as described earlier (El-Salhy et al., 1997). Briefly, a Quantimet 500MC image processing and analysis system (Leica, Cambridge, England) was connected to an Olympus microscope (type BX50), and was run with QWIN (version 1.02, Leica), a computerized image analysis program together with QUIPS (version 1.02, Leica), an interactive programming system. Quantifications were performed with x4, x20 and x40 objectives, where each pixel of the computer image corresponded to 2.12, 0.414 and 0.206 μm, respectively, and each field represented a tissue area of 1.3, 0.04 and 0.009 mm2, respectively.

The relative volume density of nerve fibres in muscularis propria was quantified in an automated standard analysis sequence according to the classical stereological point-counting method (Weibel and Elias, 1967; Weibel et al., 1969) as adapted for computerized image analysis (El-Salhy et al., 1997; El-Salhy and Spångéus, 1998a). Briefly, a 400-point lattice was superimposed on the frame covering muscularis propria. All points not covering muscularis propria were manually erased by using the computer 'mouse'. Points covering immunostained nerve fibres were marked, and a ratio of points covering nerve fibres and muscle tissue was tabulated. Measurements were performed in 20 randomly chosen fields (from 2-4 different sections, 150

<table>
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<th>ANTIGEN*</th>
<th>DILUTION</th>
<th>CODE</th>
<th>SOURCE</th>
</tr>
</thead>
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<tr>
<td>Protein gene product 9.5 (PGP 9.5)</td>
<td>1:600</td>
<td>RA 95101</td>
<td>Ultra Clone Ltd., England</td>
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<td>Synthetic substance P</td>
<td>1:1000</td>
<td>B 069082</td>
<td>BioGenex Laboratories, USA</td>
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<td>Porcine vasoactive intestinal peptide (VIP)</td>
<td>1:2000</td>
<td>R 7854/01-B5</td>
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<tr>
<td>Synthetic neuropeptide Y (NPY)</td>
<td>1:1000</td>
<td>R 840403/B6</td>
<td>Euro-diagnostica</td>
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<tr>
<td>Synthetic galanin 1-29</td>
<td>1:600</td>
<td>R 84160/B2</td>
<td>Euro-diagnostica</td>
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<tr>
<td>Synthetic rat vesicular acetylcholine transporter (VACHT)§</td>
<td>1:1000</td>
<td>R 9690/B26-1</td>
<td>Transduction Laboratories, Denmark</td>
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<tr>
<td>Human neuronal nitric oxide synthase (nNOS)</td>
<td>1:400</td>
<td>N 31030</td>
<td>Chemicon International Inc., USA</td>
</tr>
<tr>
<td>Bovine dopamine-b-hydroxylase</td>
<td>1:50</td>
<td>360PES02</td>
<td></td>
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*: all the antisera were raised in rabbit; §: specific for C-terminal

Table 1. Primary antisera used.
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The number of immunoreactive neurons in myenteric ganglia was measured using the tool kit from the manual menu. The immunolabelled neurons in the ganglion were counted manually, followed by measurement of ganglion area by drawing around the ganglion with the computer 'mouse'. From each gastrointestinal segment, ten ganglia per animal and neurotransmitter were randomly selected and measured.

The number of myenteric ganglia per mm baseline of muscularis propria was measured using the x4 objective and tools from the manual menu. Three sections from each mouse were measured by drawing a line parallel to the muscle layer and counting the number of ganglia. Ganglion area was measured by drawing a line around the ganglion, using the x20 objective. For each mouse, 15 randomly-chosen ganglia were measured (from 2-4 different sections, 150 μm apart).

Thickness of muscularis propria was measured under the x20 objective, using the manual menu, as described earlier (Spingéus and El-Salhy, 1998b). Briefly, in each mouse and each gastrointestinal segment, four fields were chosen at random, and in each field 15 perpendicular lines were drawn through the muscularis propria.

**Statistical analysis**

Comparisons between diabetic and control mice were performed with the non-parametric, Wilcoxon (Mann-Whitney) test. P-values below 0.05 were considered significant.

**Results**

**Immunocytochemistry**

Antrum, duodenum and colon of both diabetic and control mice showed nerve fibres immunoreactive to PGP 9.5, substance P, VIP, NPY, galanin, VAChT and NOS. Dopamine-β-hydroxylase-IR nerve fibres were seldom encountered in the myenteric plexus of gut, and no further quantification was made. Nerve fibres of all bioactive substances investigated were evenly distributed within muscularis propria. Whereas PGP 9.5, substance P-, VIP-, NPY-, galanin- and NOS-IR neurons were detected in the myenteric ganglia, no VAChT-IR neurons could be detected.

The antisera used in the present study immunostained nerve elements in human gut as well as dopamine-β-hydroxylase in paravertebral ganglia from mice. No immunostaining was detected when the primary antisera were pre-incubated with the corresponding bioactive substance or when the antisera were replaced by non-immune rabbit serum. Pre-

![Relative volume density of protein gene product 9.5-immunoreactive nerve fibres in the muscularis propria of antrum, proximal duodenum and distal colon of diabetic and control mice (mean ± SEM). VIP: vasoactive intestinal peptide; NPY: neuropeptide Y; NOS: nitric oxide synthase; *: p<0.05; **: p<0.01.

Fig. 1. Relative volume density of protein gene product 9.5-immunoreactive nerve fibres in the muscularis propria of antrum, proximal duodenum and distal colon of diabetic and control mice (mean ± SEM).

![Relative volume density of immunoreactive nerve fibres in the muscularis propria of antrum, proximal duodenum and distal colon of diabetic and control mice (mean ± SEM). VIP: vasoactive intestinal peptide; NPY: neuropeptide Y; NOS: nitric oxide synthase; *: p<0.05; **: p<0.01.

Fig. 2. Relative volume density of immunoreactive nerve fibres in the muscularis propria of antrum, proximal duodenum and distal colon of diabetic and control mice (mean ± SEM). VIP: vasoactive intestinal peptide; NPY: neuropeptide Y; NOS: nitric oxide synthase; *: p<0.05; **: p<0.01.
incubation of primary antibody with structurally related peptides did not affect the immunostaining.

**Computerized image analysis**

The relative volume density of nerve fibres in muscularis propria and of neurons in myenteric ganglia is illustrated in Figs. 1-3.

**Antrum**

No difference was found between control and diabetic ob/ob mice regarding PGP 9.5-IR nerve fibres. Diabetic mice showed a decreased relative volume density of NOS-IR as well as a tendency to have a decreased density of VIP-IR nerve fibres ($p=0.0728$). No difference was found regarding substance P-, NPY-, galanin- and VACHT-IR nerve fibres. The number of VIP-IR neurons/ganglionic area was significantly lower in myenteric ganglia from diabetic mice than in the controls (Fig. 4). No difference was found regarding substance P-, NPY-, galanin- and NOS-IR neurons/ganglionic area.

**Duodenum**

Density of PGP 9.5-IR nerve fibres did not differ between the two groups. Diabetic mice showed a significantly decreased volume density of VIP- (Fig. 4), NOS- and galanin-IR nerve fibres. No difference was found between diabetic and control mice regarding density of substance P-, NPY- and VACHT-IR nerve fibres. There was a decreased number of VIP-IR neurons/ganglionic area as well as a tendency to have a decreased number of NOS-IR ($p=0.0530$) neurons/ganglionic area in myenteric ganglia of diabetic mice, compared with controls. No difference was found in the numbers of substance P-, NPY- and galanin-IR neurons/ganglionic area.

**Colon**

No difference was found regarding PGP 9.5-IR nerve fibres. In diabetic mice there was a significantly increased volume density of NPY- and VACHT-IR nerve fibres. No difference was found regarding substance P-, VIP-, galanin- and NOS-IR nerve fibres. There was a tendency to have a decreased number of galanin-IR neurons/ganglionic area in myenteric plexus ($p=0.0530$). No difference was found regarding substance P-, VIP-, NPY- and NOS-IR neurons/ganglionic area.

There was no difference between diabetic ob/ob mice and lean controls regarding number of ganglia per mm baseline of muscularis propria, or of ganglion area, in antrum, duodenum and colon. Nor was there any difference regarding the thickness of muscularis propria in antrum, proximal duodenum and distal colon (Table 2).

**Table 2.** Morphometric measurements of muscularis propria thickness and of myenteric ganglia (mean±SEM).

<table>
<thead>
<tr>
<th></th>
<th>CONTROL MICE</th>
<th>DIABETIC MICE</th>
<th>P VALUE</th>
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<tbody>
<tr>
<td><strong>Antrum</strong></td>
<td></td>
<td></td>
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<tr>
<td>Muscularis propria (μm)</td>
<td>305±14</td>
<td>324±23</td>
<td>0.66</td>
</tr>
<tr>
<td>Ganglion area (mm²)</td>
<td>833±95</td>
<td>1140±216</td>
<td>0.39</td>
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<tr>
<td>Ganglion density*</td>
<td>15.5±1.5</td>
<td>13.0±1.0</td>
<td>0.24</td>
</tr>
<tr>
<td><strong>Duodenum</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscularis propria (μm)</td>
<td>42.7±4.6</td>
<td>54.3±8.8</td>
<td>0.45</td>
</tr>
<tr>
<td>Ganglion area (mm²)</td>
<td>331±38</td>
<td>365±40</td>
<td>0.63</td>
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<tr>
<td>Ganglion density*</td>
<td>6.9±0.3</td>
<td>7.5±0.7</td>
<td>0.37</td>
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<tr>
<td><strong>Colon</strong></td>
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<tr>
<td>Muscularis propria (μm)</td>
<td>121±3.0</td>
<td>110±8.6</td>
<td>0.16</td>
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<tr>
<td>Ganglion area (mm²)</td>
<td>867±211</td>
<td>798±134</td>
<td>0.90</td>
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<tr>
<td>Ganglion density*</td>
<td>10.3±0.7</td>
<td>9.6±0.8</td>
<td>0.54</td>
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</table>

*: number of ganglia per mm of longitudinal muscle.

![Fig. 3. Immunoreactive neurons per mm² of myenteric ganglia in antrum, proximal duodenum and distal colon of diabetic and control mice (mean±SEM). Abbreviations and symbols as in Fig 2.](image-url)
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Fig. 4. VIP-immunoreactive nerve fibres in the duodenal muscularis propria of a control mouse (A) and of a diabetic ob/ob mouse (B). VIP-immunoreactive neurons in antral myenteric ganglion of a control mouse (C) and of a diabetic ob/ob mouse (D). x 420
Discussion

The present investigation revealed abnormalities of several neurotransmitters in the neurons of myenteric plexus and nerve fibres of muscularis propria in obese (ob/ob) diabetic mice, an animal model of human type 2 diabetes. These abnormalities were found in all parts of gut investigated, namely the antrum, duodenum and colon. The nature of these abnormalities varied, however, between different segments of the gastrointestinal tract. Thus, whereas there was a decrease in some neurotransmitters in the upper gastrointestinal tract, there was mostly an increase in the lower gastrointestinal tract.

Although nerve fibres immunoreactive to several neurotransmitters were decreased in muscularis propria of the antrum and duodenum of diabetic mice, the total amount of nerve fibres as detected by PGP 9.5 did not differ from that of controls. This might be due to unchanged or increased nerve fibres of neurotransmitters not investigated here.

The decreased number of VIP-IR neurons/ganglionic area in myenteric ganglia and decreased relative fibre volume density in the myenteric plexus in antrum of diabetic mice observed here tallies with results of radioimmunoassays, RIA, reported earlier (El-Salhy, 1998). The present finding of a decreased number and density of VIP-IR nerves in duodenum, as well as an unaltered number and density in colon, disagrees with previously reported observations that the VIP content was unaltered or even increased in small intestine, and was decreased in colon of animal models of type 2 diabetes (Baily et al., 1986; El-Salhy, 1998). In type 1 diabetes animal models, changes in VIPergic innervation have been reported, though the nature and extent of these varies between different studies (Ballman and Conlon, 1985; Service et al., 1985; Loesch et al., 1986; El-Salhy and Spangéus, 1998b; Di Giulio et al., 1989; Belai et al., 1991; Spangéus et al., 2000). Thus, both the chemically-induced and the genetically-prone diabetes states seem to affect VIP-IR nerves of the gut.

NOS-IR fibre nerves showed a decreased relative volume density in both antrum and duodenum of diabetic mice. Furthermore, the number of NOS-IR neurons showed a tendency to affect VIP-IR nerves of the gut.

The present study showed an increased volume density of VACHT-IR fibre nerves in the colon, but an unchanged density in antrum and duodenum. This is in contrast to previous observations made in type 1 diabetes models, which showed a decreased colonic acetylcholine content, but an increased content in antrum and duodenum (Schmidt et al., 1981; Spangéus et al., 2000).

The present finding of an increased density of NPY-IR fibres in the colon of diabetic mice is in contrast to earlier RIA studies on ob/ob mice, where a decreased content of colonic NPY was found (El-Salhy, 1998). This discrepancy may be due to the fact that in the earlier study (El-Salhy, 1998) NPY was measured in extraction of whole-wall biopsies, whereas the present study investigated nerve fibres exclusively in muscularis propria.

The tendency to have a decreased number of colonic galanin-IR neurons in myenteric ganglia is in agreement with a previously observed decreased galanin content in colon (El-Salhy, 1998).

The present study shows that the neurotransmitter homeostasis is affected in an animal model of human type 2 diabetes. It is possible that the present findings may have some relevance for the gastrointestinal dysfunctions seen in patients with type 2 diabetes.

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References


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