Immediate insulin treatment prevents gut motility alterations and loss of nitrergic neurons in the ileum and colon of rats with streptozotocin-induced diabetes

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ABSTRACT

The streptozotocin-induced diabetic rat model was used to investigate the relation between the deranged gut motility and the segment-specific quantitative changes in the nitrergic myenteric neurons. Additionally, we studied the effectiveness of early insulin replacement to prevent the diabetes-induced changes.

Rats were divided into three groups: controls, diabetics and insulin-treated diabetics. Ten weeks after the onset of diabetes, animals were chosen from each group for intestinal transit measurements. The remainder were killed and gut segments were processed for NADPH-diaphorase histochemistry and HuC/HuD immunohistochemistry.

The diabetic rats displayed faster transit than that for the controls. In the insulin-treated group, the transit time was the same as that in the controls. In the duodenum of the diabetic rats, the number of nitrergic neurons was decreased, while the total neuronal number was not altered. In the jejunum, ileum and colon, both the total and the nitrergic neuronal cell number decreased significantly. Insulin treatment did not prevent the nitrergic cell loss significantly in the duodenum and jejunum, but it did prevent it significantly in the ileum and colon.

These findings comprise the first evidence that the nitrergic neurons located in different intestinal segments exhibit different susceptibilities to a diabetic state and to insulin treatment.

1. Introduction

The alterations in gastrointestinal motility, including diarrhea, observed in diabetic patients pose a major clinical problem [1,2], they appear to be multifactorial in origin. The changes include the dysfunction of extrinsic and/or intrinsic neurons in the enteric nervous system [3,4]. It has recently been demonstrated that the nitrergic subpopulation of myenteric neurons are especially susceptible to the development of neuropathy in diabetes [5–7]. Since nitric oxide (NO) is considered to be an important inhibitory neurotransmitter in the gut, impairment of the nitrergic innervation in animal models of diabetes may lead to the observed motility disorders [8,9]. Nitrergic neuropathy has been shown to take place in two phases [10]. The first phase, with the loss of neuronal nitric oxide synthase (nNOS) in the neurons and a nitrergic

dysfunction, is reversible on insulin replacement. The second phase is characterized by neuronal apoptosis and is irreversible on insulin replacement. Additionally, the preventive effect of insulin replacement on nitrergic neuron loss has been shown to be organ-dependent: it restored the decreased NOS expression in the central nervous system, but failed to do so in the kidney [9]. The data on the alterations in the nitrergic neurons in different species and in the different segments of the gastrointestinal tract are controversial. A reduced expression of nNOS and/or an impairment of NO-dependent relaxation have been reported in the myenteric plexus of the stomach [8,10–12], the duodenum [12], the colon [13] and the human jejunum [14], whereas an increased expression and thickening of nNOS-containing nerve fibers with increases in the nNOS activity and the number of nitrergic neurons have been observed in the ileum [15,16] of streptozotocin (STZ)-diabetic rats. The number of nNOS-immunoreactive myenteric neurons has been reported to be unaffected in the gastric antrum and duodenum of diabetic rats [17] and in the ileum of STZ-treated guinea pigs [18]. In consequence of these contradictory data, the mechanism through which the nitrergic myenteric neurons are involved in the diabetic complications is at present unclear.

The primary aim of the present study, therefore, was to investigate the associations between the diabetes-induced motility disorders and the alterations in the nitrergic myenteric neurons in the different segments of the small intestine and colon of the STZ-induced diabetic rat model [4,19]. A secondary aim was to study the preventive effect of early insulin replacement on the nitrergic neurons in different gut regions and the relationship with the altered gut motility.

To follow the quantitative changes in the myenteric neurons, the Plexus Pattern Analysis software developed in our laboratory [20] was applied. This has previously proved to be a reliable tool for analysis of the quantitative changes in the myenteric plexus under pathological conditions [21].

It is well established that within the enteric nervous system (ENS) the nNOS corresponds to nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) [22,23]. To identify the nitrergic neurons in the present study, therefore, we used NADPH-d histochemistry [24]. Additionally, to determine the total number of myenteric neurons, anti-HuC/D was utilized as a pan-neuronal marker [25].

2. Materials and methods

2.1. Experimental animals and induction of diabetes

All the experimental and animal care procedures had received approval from the Local Ethics Committee for Animal Research Studies at the University of Szeged. Adult male Wistar rats weighing 350–400 g, kept on standard laboratory chow (LATI, Hungary) and with free access to drinking water, were used in the study. The rats were divided into three groups: age-matched controls ($n = 10$), STZ-induced diabetics ($n = 12$) and STZ-induced diabetics with insulin replacement ($n = 12$). The controls were treated with vehicle, while diabetes was induced by a single intraperitoneal injection of STZ (Sigma, USA) at 60 mg/kg [19]. Forty-eight hours later, the

nonfasting blood glucose concentration was determined in blood obtained from the cut tip of the tail by the glucose oxidase method, using a portable blood glucose monitoring device (D-Cont Personal, 77 Elektronika Kft., Hungary). The animals were considered diabetic if the nonfasting blood glucose concentration was higher than 18 mM. From this time on, one group of hyperglycemic rats received a subcutaneous injection of 2 U of insulin (Humulin M3, Lilly, USA) each morning. Equivalent volumes of saline were given subcutaneously to the rats in the other diabetic and the control groups. Nonfasting blood glucose levels were determined, and the animals were weighed weekly in each group.

Ten weeks after the onset of diabetes, eight animals were randomly chosen from each group and prepared for intestinal transit measurements, while all the remainder were killed by cervical dislocation and gut segments were processed for morphological studies.

2.1.1. Assessment of small and large bowel transit and measurement of intestinal weights
First the colonic, and 2 days later the small bowel transit was assessed. The animals were fasted for 12 h before the measurements, though water was allowed *ad libitum* until 2 h before the beginning of the transit studies.

The colonic transit was measured according to the method described by Million et al. [26]. Briefly, a single 5-mm colored plastic bead was inserted into the distal colon (3 cm past the anus) with a plastic rod while each rat was under brief isoflurane anesthesia. After the bead insertion, the conscious rats were placed into individual cages without water or food. The time required for expulsion of the bead (in min) was monitored over 4 h.

The small bowel transit was measured by means of a phenol red content assay as described previously [27]. The animals received 2 ml phenol red (0.75 mg/ml) via an orogastric cannula. The phenol red solution was enriched with 1.5% methylcellulose, resulting in a semi-liquid bolus. Thirty minutes later, the rats were sacrificed by cervical dislocation. The abdomen was opened via a midline incision and clamps were placed at the cardia, the pylorus and the small intestine at the cecum. The small bowel was removed and laid flat, its length was measured, and it was then divided into four equal segments. The distal 10-cm segment of the ileum was cut, rinsed in water, dried on soft paper tissue and weighed. The intestinal segments were placed into separate tubes and the phenol red contents were determined spectrophotometrically as described previously [27]. The colon was also removed and laid flat, and the 10-cm segment proximal to the cecum was cut, rinsed in water, dried on soft tissue paper and weighed.

2.1.2. Histomorphological studies of the gut samples
For morphological examinations, small intestinal and colon segments were dissected and rinsed in 0.05 M phosphate buffer (PB), pH 7.4. Samples were taken from the duodenum 1 cm from the pylorus, from the jejunum 5 cm distal to the ligament of Treitz, from the ileum 5 cm proximal to the ileo-cecal junction, and from the middle part of the colon. Codes were assigned to the samples for purposes of blinded histological evaluation. The gut segments were cut along

the mesentery, pinched flat, and fixed overnight at 4 °C in 4% paraformaldehyde solution buffered with 0.1 M PB. The samples were then washed, and whole-mount preparations were made and processed for NADPH-d histochemistry, HuC/HuD immunohistochemistry and double staining with the two markers. In all cases, tissues from insulin-treated (n = 4) and untreated diabetic (n = 4) rats were processed in parallel with those from age-matched controls (n = 2).

2.2. NADPH-d histochemistry

Whole-mount preparations were processed for NADPH-d histochemistry by following the modified protocol of Scherer-Singler et al. [24]. The whole-mounts were incubated in a solution containing nitroblue tetrazolium (0.25 mg/ml), NADPH (1 mg/ml; Sigma, USA) and Triton X-100 (0.5%) in PB buffer (0.1 M, pH 7.6) for 45 min at 37 °C. The whole-mounts were mounted on gelatin-coated slides in glycerol-PB, observed and photographed with a Zeiss Axiovert 200 M light microscope equipped with an Axiocam HRc camera. Twenty digital photographs of identical magnification, size and resolution were taken from each intestinal segment. Data from 20 measured areas per animal per intestinal segment were included in the present study. Stained cells in each digital photograph were counted through use of the Plexus Pattern Analysis software [20].

2.3. HuC/HuD immunohistochemistry and double labeling with NADPH-d–HuC/HuD

Whole-mount preparations were immunostained as described earlier [21]. For double labeling, whole-mounts stained previously for NADPH-d were further processed for immunohistochemistry. All incubations were carried out at room temperature. After blocking of the preparations in PB containing 0.1% bovine serum albumin, 10% normal goat serum and 0.5% Triton X-100, they were incubated overnight with anti-human neuronal protein HuC/HuD developed in mouse (Molecular Probes, Eugene, OR; final dilution 1:20). After washing in PB, whole-mounts were incubated with biotinylated anti-mouse IgG (Amersham, Buckinghamshire, UK; final dilution 1:100) for 4 h, followed by overnight incubation in streptavidin-biotinylated horseradish peroxidase (Amersham; final dilution 1:100). Peroxidase activity was revealed by means of diaminobenzidine. Negative controls were performed by omitting the primary antibody, when no immunoreactivity was observed.

Whole-mounts were mounted on gelatin-coated slides in glycerol-PB, observed and photographed as described above.

2.4. Statistical analysis

The small bowel transit was characterized by the extent of phenol red recovery from the intestinal segments. The data obtained from the age-matched controls were compared with those from the untreated and the insulin-treated diabetics. The results are expressed as means ± S.E.M. and the statistical significance of differences between groups was calculated by ANOVA and Dunnett's test.

The results of the immunohistochemical readings were expressed as means ± S.E.M. To evaluate the effects of STZ treatment and insulin replacement on the number of nNOS-stained and HuC/HuD-stained neurons, the statistical analysis was performed by using one-way ANOVA and Newman–Keuls test. All analyses were performed by using the SPSS 8.0 software package for Windows, and a probability $P < 0.05$ was set as the level of significance.

3. Results

3.1. Immediate insulin treatment moderated the increase in nonfasting blood glucose concentration and prevented the body weight loss in STZ-induced diabetic rats

All of the diabetic rats used in this study displayed significantly increased blood glucose levels. The nonfasting blood glucose concentration in the untreated diabetic group was about four times higher than that for the controls (Table 1). In the insulin-treated group, the glucose levels on average remained below 10 mM, but this was still significantly higher than that for the nondiabetic controls (Table 1). The untreated diabetic rats lost weight significantly, whereas both the insulin-treated group and the controls gained weight during the study period (Table 1). Moreover, the weights of the corresponding segments of the ileum and colon in both the insulin-treated and the untreated diabetic rats were significantly greater than those in the control rats (Table 1). Immediate insulin replacement, however, significantly decreased the weights of the ileum and colon as compared with the untreated diabetic values (Table 1).

Table 1 – Changes in body weight, nonfasting blood glucose concentration, length of small bowel and weights of ileum and colon in control, untreated diabetic and insulin-treated diabetic rats

	Control (n = 10)	Diabetic untreated (n = 12)	Diabetic insulin-treated (n = 12)
Body weight (g)			
Initial	370 ± 27	378 ± 30	373 ± 22
Final	525 ± 22[*]	322 ± 41[*]	508 ± 40[*]
Average blood glucose (mM/l)	5.3 ± 0.8	23.8 ± 5.1[*]	8.6 ± 1.9[*]
Small bowel length (cm)	112 ± 6.8	145 ± 7.4[*]	119 ± 7.7
Ileum weight (mg/10 cm)	775 ± 46	1635 ± 173	950 ± 127[*]
Colon weight (mg/10 cm)	857 ± 71	1394 ± 105[*]	1279 ± 117[*]

[*] Statistical significance at a level of $P < 0.05$.

3.2. Immediate insulin replacement completely prevented the STZ-induced acceleration of the small intestinal and colonic transit

The distal colonic transit, measured in terms of the bead expulsion time, was significantly faster in the untreated diabetic group than in the control group (79 ± 39 min vs. 115 ± 36 min; $P < 0.05$), while in the insulin-treated group the colonic transit was not altered (Fig. 1A).

As compared with the control group, a significant shift to the right was observed in the percentages of recovered phenol red in the untreated diabetic rats, with a decrease in the first ($12.7 \pm 2.5\%$ vs. $7.3 \pm 2\%$; $P < 0.001$) and an increase in the fourth ($6.2 \pm 2\%$ vs. $25.5 \pm 10.9\%$; $P < 0.001$) segment (Fig. 1B). This indicated an accelerated small intestinal transit in the
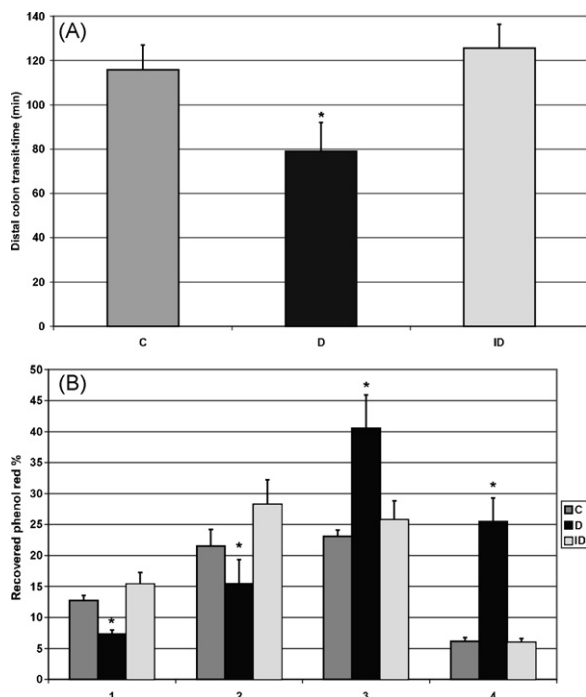
Fig. 1 – (A) Insulin replacement prevented the increase in colonic transit in rats with STZ-induced diabetes. The bead expulsion time was significantly shorter in the untreated diabetic group (n = 8) than in the control group (n = 8). In the insulin-treated group (n = 8), the colonic transit did not differ from that in the controls. Data are given as means ± S.E.M.; an asterisk indicates statistical significance at a level of P < 0.05. (B) Insulin replacement prevented the acceleration of small intestinal transit in rats with STZ-induced diabetes. As compared with the control values (n = 8), the percentage of recovered phenol red in the untreated diabetic rats (n = 8) was significantly decreased in the first and second intestinal segments, whereas it was significantly increased in the third and fourth intestinal segments, which indicates an accelerated small intestinal transit. The distribution of the recovered phenol red did not differ significantly between the controls and the insulin-treated group (n = 8). Data are given as means ± S.E.M.; an asterisk indicates statistical significance at a level of P < 0.02.
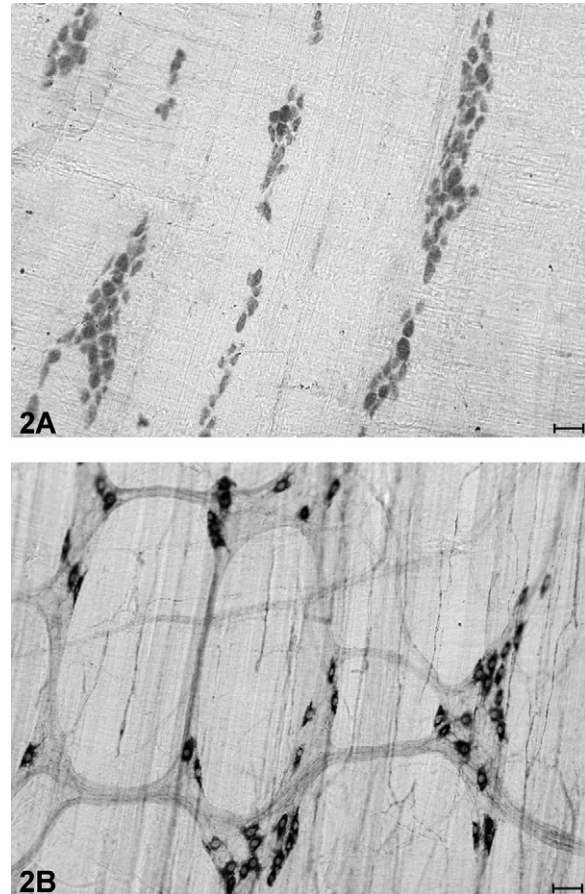
Fig. 2 – Representative micrographs of anti-human HuC/ HuD-immunostained (A) whole-mount preparations of the myenteric plexus of the duodenum from control rats, used to count the total neurons, and NADPH-d-stained (B) whole-mount preparations from the same intestinal segment, used to count the nitrergic neurons. The calibration bar denotes 50 μm in both micrographs.

untreated diabetic rats. The distribution of the percentages of the recovered phenol red among the intestinal segments did not differ significantly between the control and the insulin-treated groups, as an indication of a preventive effect of insulin treatment (Fig. 1B).

3.3. The influence of immediate insulin replacement on the densities of the total and nitrergic myenteric neurons was dependent on the intestinal segment under investigation

The densities of HuC/HuD-immunoreactive and NADPH-d-stained myenteric neurons were evaluated in the duodenum, jejunum, ileum and colon in each group of rats. HuC/HuD was used as a pan-neuronal marker (Fig. 2A), while NADPH-d histochemistry was applied to label nitrergic myenteric neurons (Fig. 2B). The NADPH-d-stained cells were considered to be nitrergic neurons when they were double-labeled with HuC/HuD (Fig. 3). With the exception of the duodenum, the density of the total myenteric neurons was decreased significantly in all intestinal segment of the untreated diabetic group of rats as compared with the vehicle-treated control
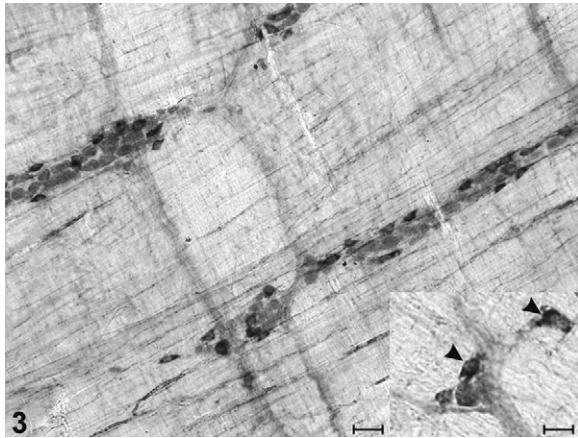
Fig. 3 – Representative micrographs of whole-mount preparations of the myenteric plexus of the duodenum from control rats double-labeled with HuC/HuD and NADPH-d. All nitrergic neurons were double-labeled for the two markers (inset). The calibration bar denotes 50 μm in the whole picture and 30 μm in the inset.

Fig. 5 – Densities of NADPH-d-positive myenteric neurons in the duodenum, jejunum, ileum and colon of control (C), diabetic (D) and insulin-treated diabetic (ID) rats. Data are expressed as means $\pm$ S.E.M. Significant differences between the control and the treated groups are indicated by asterisks. ***$P < 0.001$. Significant differences between the insulin-treated and the untreated diabetic group are indicated by circles. ○$P < 0.05$, ○○○$P < 0.001$.

group (Fig. 4). The decrease in total myenteric neuronal density was most pronounced in the jejunum (~50%; $P < 0.001$). In contrast, the density of the total myenteric neurons in the duodenum was practically the same in each group of rats. The insulin replacement partially prevented the decrease in total neuronal density in the jejunum and ileum, while it completely prevented it in the colon (Fig. 4). The density of nitrergic myenteric neurons was significantly lower (35–45%) in all intestinal segments in the untreated diabetic group of rats as compared with the vehicle-treated control group (Fig. 5). The decrease in the nitrergic myenteric neuronal density was not prevented significantly in the duodenum and jejunum after insulin replacement, whereas in the ileum and colon the prevention was significant in the insulin-treated diabetics (Fig. 5).
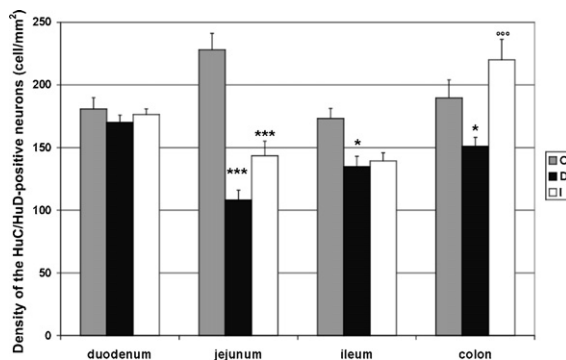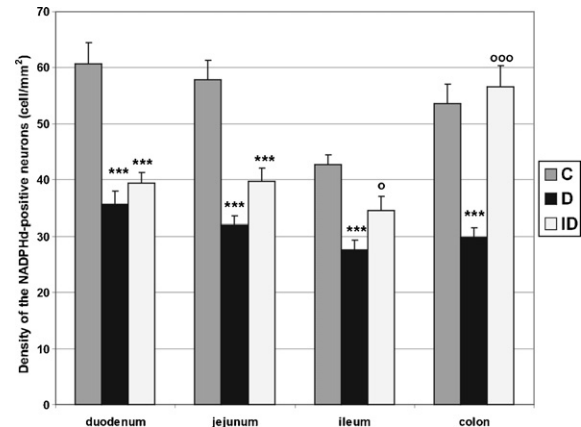

Fig. 4 – Densities of HuC/HuD-immunoreactive myenteric neurons in the duodenum, jejunum, ileum and colon of control (C), diabetic (D) and insulin-treated diabetic (ID) rats. Data are expressed as means $\pm$ S.E.M. Significant differences between the control and the treated groups are indicated by asterisks. *$P < 0.05$, ***$P < 0.001$. Significant differences between the insulin-treated and the untreated diabetic group are indicated by circles. ○○○$P < 0.001$.

4. Discussion

The two major parameters characterizing the diabetes in the STZ-induced diabetic rats were weight loss and hyperglycemia. The insulin-treated diabetic rats exhibited an increase in body weight during the study period which was similar to that for the control animals, without any significant difference at the end. Their blood glucose level was significantly lower than that of the untreated diabetics, though all of the insulin-treated diabetic rats were still hyperglycemic. Literature findings [19,28] suggest that this hyperglycemia might be responsible for the intestinal hyperplasia we observed despite insulin replacement.

The STZ-induced diabetic rats displayed faster small intestinal and colonic transit, as observed by others in different rat models of diabetes [5,29,30]. We therefore infer that our observations in this model with regard to the changes in the total myenteric neurons and the nitrergic subpopulation furnish data on the pathogenesis of diabetic diarrhea, which is a serious complication of diabetes in approximately 10% of diabetic patients [1,2].

In the gastrointestinal tract of STZ-induced diabetic rats, nNOS-containing neurons appear to be vulnerable to the effects of diabetes, but the available reports focusing only on separate segments of the gastrointestinal tract are somewhat contradictory. In rat models of diabetes, both increases [15,16] and decreases [29,31] in NOS protein expression and/ or the impairment of NO-dependent relaxation have been reported.

It is well established that NADPH-d is a reliable marker for nNOS-containing neurons in the mammalian [22] and avian [23,32] gut. In the present study, the NADPH-d-stained cells were considered to be nitrergic neurons when they were double-labeled with Huc/HuD used as a pan-neuronal marker [25]. In our experiments, the diabetic rats demonstrated a

reduction in the density of enteric neurons involving the nitrergic neurons in the myenteric plexus; however, the susceptibilities to the diabetic state and also to insulin treatment of the nitrergic neurons in the different intestinal regions varied distinctly. While the densities of both the total and the nitrergic neurons decreased significantly in the jejunum, the decrease in neuronal density in the duodenum was restricted exclusively to the nitrergic neurons. Since the total neuronal density indicated by Huc/HuD staining did not change in the duodenum of the untreated diabetic rats as compared with the control group, we concluded that the myenteric neurons did not die in this particular gut segment. Furthermore, it is well established that NADPH-d corresponds completely to nNOS within the ENS [22], and NADPH-d activity in the nitrergic neurons coincides with the nNOS activity [33]. Accordingly, we suggest that the decreased density of nitrergic neurons in the duodenum of untreated diabetic rats reflects an impairment of the nNOS pathways, rather than the necrotic or apoptotic death of the nitrergic neurons. This suggestion is consistent with our recent finding [21] of decreases in nNOS activity and nNOS protein content behind the reduced number of nitrergic myenteric neurons in an alcoholic rat model. Since the decrease in nitrergic neuronal density was the same in the duodenum of the untreated and the insulin-treated diabetic rats, we presumed that the impairment of the NOS pathways in the duodenum was independent of the insulin deficiency. Otherwise, the enhancement of the preventive effect of early insulin replacement on the decrease in density of both the total and the nitrergic myenteric neurons was well pronounced in the aboral direction. The neuronal density in the jejunum after insulin replacement was still significantly lower than that in the controls; the difference between the controls and the insulin-treated diabetics was no longer significant in the ileum; whereas the nitrergic neuronal density in the colon was the same in the insulin-treated diabetics as that in the control group. In conclusion, our present results correlate well with previously reported data which proved that the gastrointestinal malfunction in diabetes includes changes in the myenteric nitrergic neurons. However, the evidence published here has revealed for the first time the different susceptibilities of nitrergic neurons located in different intestinal segments to diabetic damage, and also their different levels of responsiveness to insulin treatment. The exact nature of these differences warrants further investigations. However, these findings imply that the development of the diabetic nitrergic neuropathy is more complicated than suggested earlier [10] and it differs from segment to segment along the gastrointestinal tract.

Conflicts of interest

The authors state that they have no conflict of interest.

Acknowledgements

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