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TERMINAL ILEUM SUBMUCOUS PLEXUS

Study of the VIP-ergic neurons of diabetic rats treated with ascorbic acid

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ABSTRACT - The aim of this study was to evaluate the effect of the ascorbic acid (AA) supplementation on the neurons that produce the vasoactive intestinal peptide (VIP) in the submucous plexus of the ileum of rat, four months after the induction of experimental diabetes mellitus with streptozotocin. Three groups of rats were used: C - control, D - diabetic, DA - diabetic receiving AA. We have measured the immunoreactivity and area of 80 cellular bodies of VIP-ergic neurons from each studied group. In the diabetic animals, we have observed hyperphagia, polydipsia, and an increase of glycemia and glycated hemoglobin. The VIP-ergic neurons have presented an increase of their immunoreactivity and the highest profiles when compared to the other groups. In the diabetic animals supplemented with AA it has been observed a small reduction in the glycemia and the water and food intake. We have also noticed smaller immunoreactivity in their VIP-ergic neurons, similar to what we have observed in the control group animals (group C).

KEY WORDS: ascorbic acid, diabetes mellitus, streptozotocin, ileum, vasoactive intestinal peptide, submucous plexus, rats.

Plexo submucoso de íleo terminal: estudo dos neurônios VIP-érgicos de ratos diabéticos tratados com ácido ascórbico

RESUMO - O objetivo deste estudo foi avaliar o efeito da suplementação com ácido ascórbico (AA) sobre os neurônios que expressam o peptídeo intestinal vasoativo (VIP) no plexo submucoso do íleo de ratos, quatro meses após a indução do diabetes mellitus experimental com estreptozootocina. Três grupos de ratos foram usados: C- controles, D- diabéticos, DA- diabéticos recebendo AA. Foram avaliadas a imunoreatividade e a área de 80 corpos celulares de neurônios VIP-érgicos de cada grupo estudado. Nos animais diabéticos ocorreram hirperfagia, polidipsia, elevação da glicemia e hemoglobina glicada. Os neurônios VIP-érgicos apresentaram aumento da imunorreatividade e os maiores perfis, quando comparados aos demais grupos. Nos animais diabéticos suplementados com AA observou-se pequena redução na glicemia, ingesta de água e de alimento, verificando-se também menor imunorreatividade nos neurônios VIP-érgicos, o que foi semelhante ao observado nos animais do grupo controle (grupo C).

PALAVRAS-CHAVE: acido ascórbico, diabetes mellitus, estreptozootocina, íleo, peptídeo intestinal vasoativo, plexo submucoso, ratos.

The neurological manifestations of diabetes mellitus (DM) occur in the peripheral nervous system and particularly in the enteric nervous system. It is observed in the digestive system a dilatation of the stomach, small and large intestine[1,2]. One of its more relevant clinical problems is the diabetic diarrhea and constipation[3]. The etiology of these disturbs are not completely known. However, degenerative changes in the enteric nervous system are related to the development of the diabetic neuropathy. In a more accurate assessment of the DM effects on the enteric nerves, we have observed in previous studies, developed in our laboratories, a reduction of the number of myenteric neurons in several intestinal segments[2,4]. This pathology also promotes changes in the contents of the enteric neuropeptides such as the vasoactive intestinal peptide[5] (VIP), whose production increases significantly in the intestine of dia-

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betic rat[6]. It is believed that changes in the VIP-ergic neurons, which represent 50% of the overall neurons present in the submucous plexus[7], may imply in gastrointestinal disorders[6,8] such as the diabetic diarrhea syndrom[9].

Several studies have considered some factors as the responsible for the appearance of degenerative changes. Among the possible causes are: a) peripheral nerves lesions, due microcirculation changes (*vasa nervorum*); b) oxidative stress, which is intensified in DM; c) increased in the sorbitol level[10,11]. The oxidative stress produce free radicals, which appear due to the increase of non-enzymatic glycation, increase of self-oxidation and increase of metabolic stress[10]. Free radicals are usually neutralized by antioxidants agents such as the ascorbic acid (AA)[12]. The concentration of AA in diabetic people is reduced, since is used to neutralize the free radicals and also because its transportation is inhibited due to the appearance of hyperglycemia as well as its renal absortion[13].

The oxidative stress may also result from changes in the sorbitol formation stages. Sorbitol is produced by the glucose reduction, in the reaction catalyzed by the aldose reductase enzyme[11]. The increase of the sorbitol level promotes an increase of the intracellular osmolarity, with formation of edema, neuronal lesions and a consequent reduction of nerve conduction velocity[14]. Drugs that improve the control of the oxidative stress and decrease the sorbitol presence through the inhibition of the aldose reductase enzyme may have a relevant role in the treatment of diabetes neurological problems. The AA is one of these substances and it has been studied in the treatment of this pathology. The supplementation with AA shows little effects on the blood glycemia concentration[15,16], but it reduces the capillarity fragility and also the cellular sorbitol concentration[12,17,18], suggesting a neural protector role for this substance.

In this paper we have studied the neural protector effect of the ascorbic acid on the VIP-ergic enteric neurons of the submucous plexus in the ileum of diabetic rat.

METHODS

Animal procedures

Male Wistar rats (*Rattus norvegicus* rats) weighing 300-400g, aged around 13 weeks were employed. To induce diabetes, rats were starved for 14 hours and then streptozotocin (35 mg/Kg b.w., Sigma, USA) was injected i.v.. Non-diabetic rats were employed as control group. Streptozotocin injection resulted in a diabetic syndrome with rapid weight loss, polyuria and glycosuria. Rats were divided in 3 groups: ascorbic acid-treated diabetes (DA group), untreated diabetes (D group) and untreated control (C group). Ascorbic acid was given for 16 weeks from the onset of the diabetes by adding ascorbic acid (Sigma, USA) to drinking water (1 g/L prepared fresh each day)[16]. The animals were kept in individual metabolic cages in a room with a maintained photoperiod (6:00 a.m. – 6:00 p.m.) and room temperature (RT) (24º ± 2ºC). Water was given *ad libitum* and Nuvital® lab chow served as the diet.

Thus, 16 weeks after streptozotocin (D and DA groups) or control group, the rats were anesthetized intraperitoneally with thiopental (40 mg/kg-body wt.). Blood was collected by cardiac puncture for the measurement of glycated hemoglobin[19], glucose[20] and ascorbic acid[21] level. The rats were observed during 4 months. Water consumption, food intake and urine elimination were monitored.

Immunohistochemistry and morphological analysis

After abdominal incision the ileal segments were collected, rinsed in 0.01M phosphate buffer saline (PBS), pH 7.4, and fixed in Zamboni's liquid for 18 hours[22] at 4º C, the segments were processed according to the immunohistochemistry technique for whole-mount preparation[23] in order to detect the presence of VIP in the submucous plexus.

Briefly, segments were opened along the mesenteric border, washed and dehydrated, diaphanized in xilol and rehydrated. Afterwards were place in 0.01M PBS pH 7.4. Samples were reduced with the aid of a circular sectioner and the mucosa and muscle layers were dissected under stereomicroscope. The submucous layer isolated was incubated with polyclonal rabbit anti-VIP (Penninsula Labs, USA) overnight at RT at 1:200. The samples were washed in PBS and then incubated in sequence with the secondary FITC-conjugated antibody (Penninsula Labs, USA) for 1h at 1:100 (RT) under shaking. In control samples, the primary antibody was substituted by goat serum. The whole-mounts were placed in glycerol-coated slides.

The immunofluorescence was analyzed on a trinocular biological optic microscope, 40X lens, equipped with immunofluorescence filters (FITC) and a kit to capture images IPPWIN-DCAM. The images were taken by a high-resolution camera, transmitted to personal computer and then recorded in a compact disc.

The area (μm2) of 80 cellular bodies of immunoreactive VIP-ergic neurons (VIP-IR) from each group studied was measured through the image analysis software Image-Pro-Plus 3.0.1.

Statistical analysis

The data were analyzed by the minimum squares method, through the variance analyzes and the Tukey's test to compare the averages.

We have employed the methodology and General Linear Model (GLM)[24] and the Student's t-test to compare the averages due to the fact that area of the VIP-IR neurons cell bodies did not present a normal distribution. We have used the GAMA distribution, with functions of linking and identification. The analyses were carried out with the GLIM 4.0 software.

Table 1. Body weight, in grams/aged 90 days (BW/90) and body weight, in grams/aged 210 days (BW/210), daily water consumption (DWC), daily food intake (DFI), daily urine elimination (DUE) in untreated controls (C), untreated diabetes (D) and ascorbic acid-treated diabetes (DA) groups.

	BW 90/ g	BW210/g	DWC/ml	DFI/ g	DUE/ ml
C	338.9±6.8 (10)[a]	466.0±6.2 (10)[a]	64.9±5.9 (5)[a]	30.7±0.9 (5)[a]	3.3±0.4 (5)[a]
D	335.7±6.0 (10)[a]	318.9±4.6 (10)[b]	158.6±8.4 (5)[b]	46.9±3.3 (5)[b]	59.3±7.1 (5)[b]
DA	339.4±8.8 (10)[a]	315.6±2.9(10)[b]	133.6±5.4 (5)[c]	39.6±3.1 (5)[b]	72.4±4.0 (5)[b]

Means followed by different letters in the same column are different by Tukey test ($p < 0.05$). All results were expressed as mean ± SE. (n) = number of rats.

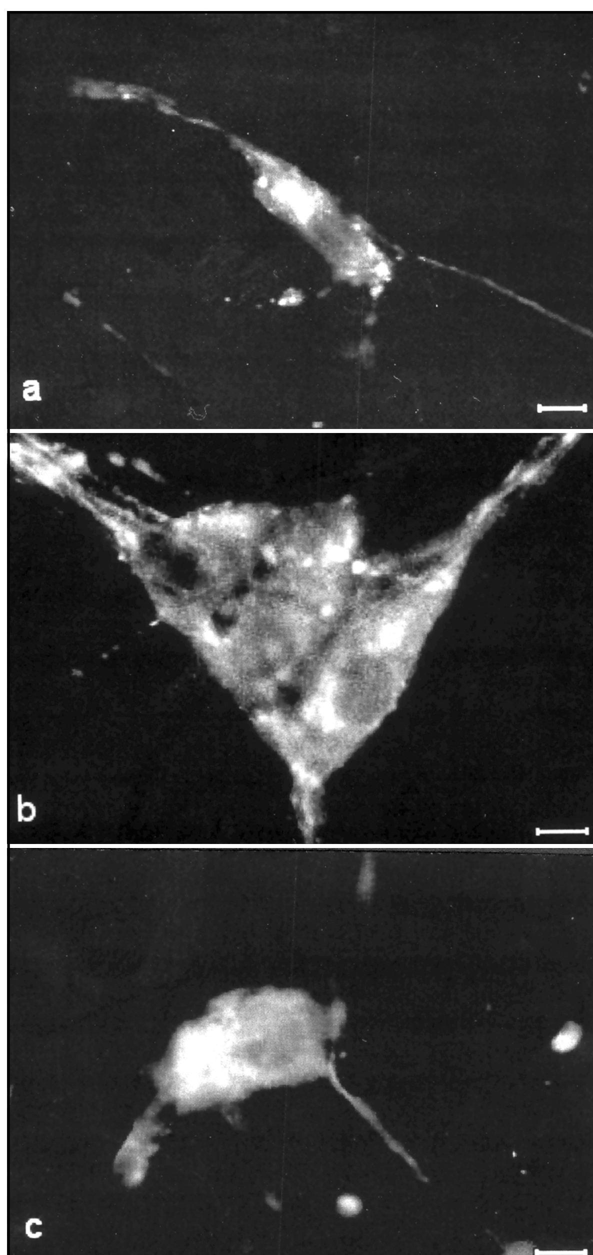

Fig 1. Immunofluorescence micrographs showing VIP immunoreactivity in the submucous plexus of ileum from untreated controls (a), untreated diabetes (b) and ascorbic acid-treated diabetes (c) groups. Calibration bars = 10 µm.

RESULTS

Streptozotocin promoted a diabetic syndrome, since hyperphagia, polydipsia, polyuria and loss of body weight were observed (Table 1). However, the results for D and DA groups were similar.

Diabetes control and plasmatic ascorbic acid concentration level

The acid ascorbic supplementation reduced the glucose blood level in rats from group DA ($p < 0.05$). However, we have not noticed differences in the glycated hemoglobin level among the groups of diabetic rats. (Table 2)

The supplementation increased the plasmatic ascorbic acid level in the DA group ($p < 0.05$) when compared to group D (Table 2).

Immunohistochemistry and morphological analyses

VIP-IR cell bodies were observed in the submucous plexus of the ileum of rats in the control group (Fig. 1a). We have showed an increase in the fluorescence intensity in nervous fibers and cellular bodies of the submucous plexus of the diabetic rats (Fig. 1b). There was no increase in the fluorescence intensity of the VIP-IR cell bodies (Fig. 1c) in the rats treated with ascorbic acid (DA group).

Deviance analyses showed that the VIP-IR cell bodies area average was different in the groups studied (Table 3). The measured areas ranged from 233.7 μm^2 (C group) to 1612.8 μm^2 (D group).

The averages of the VIP-IR cellular bodies areas are shown in Table 4. There was a significant area increase in the D group compared to the C group. The neurons from DA group presented smaller areas when compared to D group. However, this difference was not significant.

Table 2. Glycemia (GYL), glycated hemoglobin (GHb) and plasma ascorbic acid (AA) in untreated controls (C), untreated diabetes (D) and ascorbic acid-treated diabetes (DA) groups.

	GYL/mg.dl^{-1}	GHb/%	AA/µg.ml^{-1}
C	144.4 ± 6.7^{a}	4.1 ± 0.3^{a}	26.7 ± 2.7^{a}
D	506.0 ± 8.0^{b}	8.1 ± 0.2^{b}	19.1 ± 3.5^{ab}
DA	452.3 ± 10.6^{c}	7.9 ± 0.5^{b}	32.4 ± 1.8^{ac}

Means followed by different letters in the same column are different by Tukey test ($p < 0.05$). All results are expressed as mean ± SE. n= 10 rats, for group.

Table 3. Deviance analysis of means from VIP-IR neurons cell bodies area in the three studied groups.

Variance source	GL	Deviance	Mean deviation	F
Treatment	2	6.024	3.012	24.77*
Residue	237	28.833	0.1216	

*Significant to the level of 5 % of probability.

Table 4. Means and standard errors of VIP-IR neurons cell bodies areas in untreated controls (C), untreated diabetes (D) and ascorbic acid-treated diabetes (DA) groups.

Groups	Means ± SE
C	551.8 ± 20.4^{a}
D	858.1 ± 33.3^{b}
DA	791.6 ± 30.2^{b}

Means followed by different letters are different by test 't" ($p < 0.05$).

DISCUSSION

Our studies have shown that the diabetes mellitus induced by streptozotocin caused changes in the area and in the immunoreactivity of VIP-ergic neurons from the submucous plexus of the ileum of rats. Several studies have shown that the diabetes present a differentiated effect, depending on the intestinal region and on the kind of neurotransmitter considered. The most evident changes were showed on the VIP neurotransmitters, noradrenaline and serotonin, with no recorded changes for the substance P[6,25,26,27].

We have been able to verify that rats with a 16-week-old-diabetes showed an increase in the cell body area of VIP-ergic neurons in the submucous plexus when compared to the control animals. This increase may be related to the synthesis of neuropeptides in the cell since we have observed an increase in the immunoreactivity of neurons cell bodies in these animals. The immunoreactivity of neurons in the DA group was similar to that in the control group, showing that the VIP levels in the cells of DA group increased proportionally to the increase of the cellular profile. There was an intensification of the VIP levels in the D group.

Belai & Burnstock[26] also observed the increase of VIP-IR in the submucous plexus of 16-week-old diabetic rats. Biochemical evaluation, besides the morphological evaluation, proved that the VIP levels increase in the presence of diabetes[5,6]. This supports the relation between the levels of this neurotransmitter and the increase of immunoreactivity observed in the light microscopy. It has also been suggested that the increase on the area of cellular body of VIP-ergic submucous neurons may be related to the reduction on the number of neurons in the myenteric submucous since that, although the enteric plexus are spatially separated, the connection between them suggest they form an integrative unit[28]. See et al.[29] observed an increase on the cellular body volume of submucous VIP-ergic neurons after denervation myenteric. The authors speculate that in normal conditions the myenteric plexus would have an indirect inhibitory action over the submucous plexus to which it is connected. The removal of the inhibitory impulse for the submucous neurons could result in a VIP production increase, leading to an increase in the area of the submucous VIP-ergic neurons.

It has been verified, through quantitative analyses carried out with the material from the animals employed in this experiment, a reduction in the number of neurons of the myenteric plexus in groups D and DA through the Myosin-V technique (personal communication). This has also been observed previously in the small[2] and large[4] intestine of rats. Taking in consideration the results presented by See et al.[29], we can infer that the loss of myenteric neurons itself could already lead to a functional overcharge in the submucous neurons with consequences to their morphology. It may also be possible there was a specific loss of submucous VIP-ergic neurons due to the diabetes; this way, the remaining submucous and myenteric neurons would have to increase their activity to compensate for the lost neurons. This increase in their activity would be responsible for an increase in the synthesis processes, thus, the intracellular machinery would become more and more developed making the cell gain volume. Another factor to be considered (and that could also contribute to the increase of VIP synthesis by the submucous neurons) is the increase of food intake observed in diabetic animals since the liberation of this neurotransmitter may be promoted by the mucosa mechanic stimulation[7].

The increase in the neuronal profile area in diabetic rats could also be related to the intracellular edema provoked by the sorbitol accrual[14]. However, the edema contribution is not the primordial factor and we should not bestow it a higher importance than other factors: these cells presented in the DA group immunoreactivity levels similar to the C group while in the D group the higher immunoreactivity is a sign of intense synthesis activity and/or neurotransmitter accrual in the cellular body. If the primary cause of the cellular body increase of these neurons was the edema, the cytoplasmic components would be more scattered and their immunoreactivity would be less than the observed in the control animals.

It has been shown that the diabetes leads to a reduction of the circulatory levels of AA[13]. As the AA is a co-factor for the noradrenaline synthesis[30] it is possible that this neurotransmitter may not be synthesized with the same efficiency. On the other hand, the peripheral neuropathy related to the sorbitol accrual would compromise the integrity of the sympathetic neurons cell bodies and nervous fibers. This would imply in a sympathetic nervous system inhibitory action deficit in the submucous collaborating to overcharge the VIP-ergic neurons. This hypothesis would explain why although the DA group neurons have presented an increase in their cell profiles, they have expressed a lower immunoreactivity for VIP than the rats' neurons from D group. This suggested us that, in the DA group, the action of the sympathetic neurons may be more efficient, as much for the higher capacity of noradrenaline synthesis as for the better morphological conditions of the sympathetic nervous fibers that connect with the VIP-ergic submucous neurons. Since the AA is an inhibitory substance of the aldose redutase enzyme[12,17,18], which contributes to a decrease in the sorbitol production, the main agent of peripheral neuropathies in diabetic people.

Our result with the use of AA was similar to the one where ponalrestat, an aldose reductase inhibitor, was given to rats with chronic diabetes[31]. This drug, similarly to AA, prevented the increase of immunoreactivity of VIP-ergic neurons[31]. The plasmatic concentration of ascorbic acid was reduced 27.9% in the animals from the diabetic group when compared to the animals from the control group. This reduction has been related to a higher exposition of the diabetic animals to oxidative stress[16], higher urine excretion and to the hyperglycemia, which inhibits the AA transportation to the interior of some cells[12].

In human beings suffering from diabetes mellitus, the reduction of blood AA is of about 30%[12]. Perhaps, this reduction in rats happens to be smaller, for unlike human beings, these animals are able to synthesize AA[12]. The supplementation with AA in the diabetic animals (DA group) raised the plasmatic level of this substance in 41% when compared to the diabetic animals that did not receive this supplementation (D group) and in 17.6% when compared to the C group. There were also changes in the glycemic level and in the glycated hemoglobin in the diabetic animals receiving the supplementation, respectively 10.6% and 2.5% less than in the non-supplemented diabetic animals.

Summing up, we have verified the following in the non-supplemented diabetic animals: a great increase in the water and food intake; a great rise in the glycemia and glycated hemoglobin; an increase in the immunoreactivity and larger cell profile areas of the VIP-ergic submucous neurons when compared to the other groups. On the other hand, when comparing the diabetic animals supplemented with AA to the non-supplemented diabetic animals we have observed a smaller reduction in the glycemia, water and food intake and also a smaller immunoreactivity in the VIP-ergic neurons, which was similar to the observed in the animals of the control group (C group).

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