Decreased VIP Content in Peripheral Nerve From Streptozocin-Induced Diabetic Rats

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After induction of diabetes with streptozocin (STZ-D) in rats, we measured vasoactive intestinal polypeptide (VIP) content in sciatic nerve and spinal cord obtained from nondiabetic, untreated STZ-D, and insulin-treated STZ-D rats. Eight weeks after the onset of diabetes, caudal nerve conduction velocity (NCV) in the untreated STZ-D rats (n = 13) was slower than in the controls (n = 11; mean ± SE 30.9 ± 0.6 vs. 41.4 ± 1.8 m/s, $P < 0.001$). The decrease in NCV was less marked in the insulin-treated STZ-D rats (n = 11; 36.3 ± 0.9 m/s, $P < 0.05$ vs. control). VIP content in sciatic nerve decreased in the untreated STZ-D rats (1.33 ± 0.23 ng/g wet wt) compared with the other groups (control, 3.10 ± 0.44, $P < 0.01$; insulin-treated STZ-D, 2.44 ± 0.55, $P < 0.05$). However, in spinal cord, VIP content was not significantly different among the three groups. The VIP levels in sciatic nerve showed a positive correlation with NCV (r = 0.430, $P < 0.01$). In addition, an inverse correlation between VIP levels and blood glucose levels was observed (r = −0.5624, $P < 0.001$). NCV was also inversely correlated with blood glucose levels (r = −0.7662, $P < 0.001$). Together with a previous morphological study, these findings suggest a possible causal relationship between reduced VIP content and diabetic neuropathy. *Diabetes* 39:608–12, 1990

Vasoactive intestinal polypeptide (VIP), originally isolated from pork small intestine (1), has a wide range of biological activities, e.g., vasodilation and hypotension (1–4), stimulation of glycogenolysis and lipolysis (5,6), and stimulation of insulin and glucagon release (7,8). At first, VIP was considered to be a gut hormone (9), but immunohistochemical studies have demonstrated that VIP is found in neurons widely distributed throughout the body (10–14). VIP seems to act as a neurotransmitter, neuromodulator, or neurohormone in central and peripheral nervous systems, including autonomic and sensory neurons (15–19).

Immunohistochemical methods have demonstrated that VIP is also present in varicose autonomic nerve fibers in perivascular plexuses of vasa nervorum, i.e., blood vessels supplying blood to the peripheral nerves (20). Neurohumoral control of this small vessel is thought to be important in normal peripheral nerve function, and VIP might play a role in the pathogenesis of neuropathy associated with diabetes mellitus. In fact, Crowe et al. (14) and Gu et al. (21) reported that a reduction in VIP nerve innervation was observed in the penile erectile tissue of diabetic patients with impotence and streptozocin-induced diabetic (STZ-D) rats. However, these data neither proved nor disproved a cause-effect relationship between VIP and diabetic neuropathy.

The aim of our investigation was threefold. First, to compare the VIP content in the peripheral and central nervous systems in diabetes, we measured VIP content in two tissues from these nervous systems from STZ-D rats. Second, to investigate whether VIP is pathophysiologically related to diabetic neuropathy, nerve conduction velocity (NCV) was measured simultaneously with VIP as an indicator of peripheral nerve function. Third, diabetes-induced changes in these parameters were examined by linear regression analysis.

RESEARCH DESIGN AND METHODS

Male Wistar rats aged 10 wk and weighing 270–340 g were divided into three groups. One (n = 11) was a control group that was given an intraperitoneal injection of citrate-saline buffer (pH 4.5) only. Another group (n = 13) was given an injection of 60 mg/kg i.p. STZ (lot 605114, Calbiochem-Behring, La Jolla, CA) that was dissolved in citrate-saline buffer just before injection. The STZ-D rats developed glycosuria, polyuria, hyperglycemia, and weight loss. The third group

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(n = 11) comprised STZ-D rats treated with an injection of Lente insulin (4 U/day s.c.) after the onset of diabetes. Heparinized whole blood was taken from a tail vein for plasma glucose measurements by the glucose oxidase method with the Glucose Analyzer 2 (Beckman, Fullerton, CA). All rats were maintained under the same conditions and supplied with a standard food (CE-2, JCL, Osaka, Japan) with water ad libitum.

NCV. Caudal NCV was determined with a modification of the method of Miyoshi and Goto (22) with Neuropack-11 MEB-5100 (Nihon Kohden, Tokyo). In brief, before and 8 wk after onset of diabetes, all rats were anesthetized with an injection of pentobarbital sodium (50 mg/kg i.p.). The tail of each rat was kept in a thermostatically controlled liquid paraffin bath to maintain constant subcutaneous temperature at 37°C. Caudal NCV was measured by stimulating the proximal point of the tail and recording the muscle action potentials at two distal points with stainless steel needle electrodes. Electrical stimulation was a rectangular pulse, 0.2 ms in duration, with voltage sufficient to evoke a supramaximal response. NCV was calculated by dividing the distance between the two recording electrodes (exactly 5 cm) by the latency. To prevent tissue damage of the sciatic nerve before VIP assay and keep a constant subcutaneous temperature for accurate NCV measurement, we used caudal NCV as a substitute for sciatic nerve NCV.

Tissue sampling and VIP extraction. Eight weeks after the onset of diabetes, rats were killed by cervical dislocation, and the bilateral sciatic nerves were surgically removed and dissected free from extraneural connective tissue. The entire spinal cord was also removed by the method of De Sousa and Horrocks (23). In brief, two parallel longitudinal incisions were made on each side of the vertebral column at the sacral level. Transection was done at the most distal position. A syringe was filled with cold saline, and its needle (16 gauge) was inserted ~5 mm from the caudal end of the vertebral canal. The spinal cord was ejected by hydraulic pressure of saline through the syringe. The tissues were immediately weighed and stored at −80°C until the extraction of VIP.

The frozen tissues were boiled for 5 min in 10 × vol (wt/vol) 1 N acetic acid and homogenized with a Polytron homogenizer. The homogenates were centrifuged at 10,000 × g for 20 min at 4°C. VIP was then extracted from the supernatant with 75% acetone. The collected solvents were evaporated and lyophilized. Immediately before assay, the sample was resuspended in an appropriate volume of radioimmunoassay buffer as stated below. Mean ± SE overall recovery rate of VIP was 85.5 ± 1.9% (n = 6).

Radioimmunoassay of VIP. As previously reported from our laboratory (24), VIP was measured by radioimmunoassay with specific antibody R-501, which was kindly provided by N. Yanaihara (Shizuoka College of Pharmacy, Shizuoka, Japan; 25,26). The antiserum was used at a final dilution of 1:180,000. Cross-reactivity of this serum with other polypeptides, e.g., secretin, gastrin, glucagon, motilin, substance P, C-peptide, somatostatin, cholecystokinin, and pancreatic polypeptide, was substantially negligible. Pork VIP (2 μg, Peptide Institute, Osaka, Japan) was labeled for 10 min with ^{125}I-labeled sodium (0.5 mCi) with lactoperoxidase. The tracer was purified on an SPC 25 Sephadex column (1 × 10 cm) in 0.1 M ammonium acetate (pH 7.4) and eluted with 1.2 M ammonium acetate (pH 7.4) with 0.1% bovine serum albumin. The assay buffer was 0.01 M phosphate buffer (pH 7.4) with 0.5% bovine serum albumin, 0.01% EDTA, 0.14 M NaCl, and 250 KIU/ml aprotinin. After preincubation of the antibody and standard VIP or samples for 24 h at 4°C, ^{125}I-labeled VIP was added. The assay tubes were further incubated for 48 h at 4°C. Bound and free VIP were separated with dextran-coated charcoal. All samples were measured in duplicate. The intra-assay coefficient of variation was 4.4%, and the interassay coefficient of variation was 15.1%. The detection limit of this assay was 1.6 pg/tube.

Statistical analysis. All values are means ± SE. Student's t test or Welch's method after inspection of variance was used for the nonpaired or paired comparison with 5% significance level. Linear regression analysis was also used.

RESULTS

Development of diabetes. Before STZ injection, mean ± SE body weights of the three groups were not different (control, 313.2 ± 5.0 g; untreated STZ-D, 309.6 ± 4.6 g; insulin-treated STZ-D, 315.0 ± 4.4 g). However, 3 days after STZ injection, plasma glucose in the untreated STZ-D and insulin-treated STZ-D rats increased compared with controls. Long-term insulin injection effectively prevented extreme hyperglycemia and progressive weight loss in STZ-D rats (Table 1). Body weight was negatively correlated with plasma glucose levels in the experimental rats (r = −0.8955, P < 0.001).

Radioimmunoassay of VIP. The standard curve of VIP assay is shown in Fig. 1. Dilution curves of the samples from sciatic nerve and spinal cord were parallel to the standard curve of VIP.

Changes in NCV. NCV was measured twice, before and 8 wk after STZ injection (Fig. 2). Before STZ injection, no dif-

TABLE 1
Characteristics of experimental rats after streptozocin injection

		Body weight (g)		Blood glucose (mM)	
	n	After 3 days	After 8 wk	After 3 days	After 8 wk
Control	11	341 ± 6	427 ± 7	8.0 ± 0.9	9.0 ± 3.2
Untreated diabetic	13	295 ± 4*	222 ± 6*	32.1 ± 2.8*	28.2 ± 1.0*
Insulin-treated diabetic	11	308 ± 6*	357 ± 10*†	32.3 ± 2.1*	19.7 ± 1.7*†

Values are means ± SE.
*P < 0.001 vs. control rats.
†P < 0.001 vs. untreated diabetic rats.

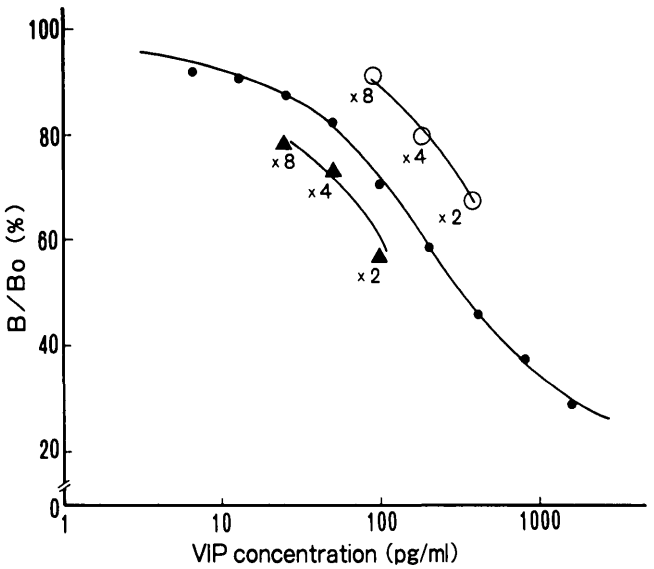

FIG. 1. Standard curve of vasoactive intestinal polypeptide (VIP). Sample was resuspended in 2 vol assay buffer. Dilution curves of extracted samples from sciatic nerve (▲) and spinal cord (○) were obtained after serial dilution of sample by 2× and 4×. B/B_0, percentage of antibody-bound ^{125}I-labeled VIP in absence of added standard VIP (B_0) against presence of added standard VIP (B).

ference in NCV was observed between the three groups (control, 27.2 ± 1.0 m/s; untreated STZ-D, 27.7 ± 0.5 m/s; insulin-treated STZ-D, 29.0 ± 0.7 m/s). After 8 wk, NCV was significantly increased in every group (control, 41.4 ± 1.8 m/s, $P < 0.001$; untreated STZ-D, 30.9 ± 0.6 m/s, $P < 0.001$; insulin-treated STZ-D, 36.3 ± 0.9 m/s, $P < 0.001$). A gradual increase in NCV with age was previously reported in rats (22,27). However, in the untreated STZ-D rats, this increase was less marked than the controls. NCV value of the insulin-treated STZ-D rats was between the NCV levels of the other two groups, indicating that insulin treatment partially improved impaired peripheral nerve function. Furthermore, there was a negative correlation between plasma glucose levels and NCV in the experimental rats ($r = -0.7662$, $P < 0.001$, data not shown).

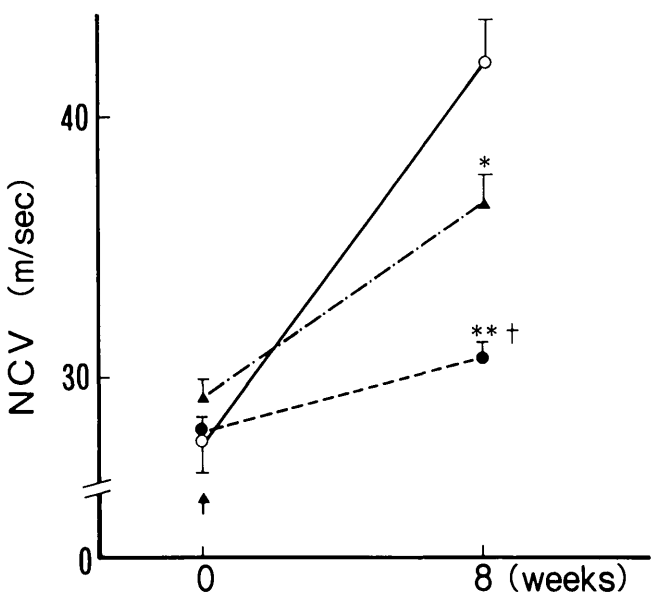

FIG. 2. Changes in nerve conduction velocity (NCV) during experiment. ○, Control rats; ●, untreated streptozocin-induced diabetic (STZ-D) rats; ▲, insulin-treated STZ-D rats. Week 0 is defined as 1 day before STZ injection (*arrow*). Values are means ± SE. *$P < 0.05$, **$P < 0.001$, vs. controls. †$P < 0.001$ vs. insulin-treated STZ-D rats.

VIP content in tissues. Eight weeks after STZ injection, wet weight of sciatic nerve and spinal cord in the untreated STZ-D rats decreased compared with controls. However, this reduction was completely prevented by the insulin treatment (Table 2).

VIP content in spinal cord was unchanged in value among the three groups (Fig. 3*A*). In contrast, VIP in sciatic nerve (Fig. 3*B*) was reduced in the untreated STZ-D rats (1.33 ± 0.23 ng/g wet wt) and restored by insulin to a level similar to that of the controls (3.10 ± 0.44 ng/g wet wt, $P < 0.01$) in the insulin-treated STZ-D rats (2.44 ± 0.55 ng/g wet wt, $P < 0.05$). VIP content in control and insulin-treated STZ-D rats was not significantly different. These results indicate that insulin treatment reversed the decreased VIP content in diabetic sciatic nerve. To examine whether loss of fat tissue found in diabetes masked a decrease in VIP content in the spinal cord, total VIP content was compared. Total VIP content in spinal cord of untreated STZ-D rats (1.61 ± 0.12 ng/total g wet wt) showed a tendency to be lower than for the other groups (control, 2.09 ± 0.22 ng/total g wet wt; insulin-treated STZ-D, 2.12 ± 0.23 ng/total g wet wt), but these differences were not significant. In addition, VIP levels in the sciatic nerve negatively correlated with plasma glucose levels ($r = -0.5624$, $P < 0.001$, data not shown).

Relationship between VIP content and NCV. To evaluate the significance of reduced sciatic nerve VIP content in the pathogenesis of diabetic peripheral neuropathy, linear regression analysis between NCV and VIP content in sciatic nerve was examined. Separate analysis for each group did not result in significant correlation ($r = 0.082$, -0.264, and 0.348 in control, untreated STZ-D, and insulin-treated STZ-D rats, respectively). However, linear regression analysis for all three groups revealed a positive correlation between NCV and VIP levels in sciatic nerve (Fig. 4). However, these results do not necessarily prove the cause-effect relationship between VIP and diabetic neuropathy.

DISCUSSION

This study demonstrates that VIP content in sciatic nerve is significantly reduced in STZ-D rats. VIP levels in sciatic nerve were negatively correlated with blood glucose levels. Insulin treatment restored the decreased VIP content in this nerve to a level similar to controls. These results suggest that hyperglycemia and/or insulin deficiency leading to other met-

TABLE 2
Wet weights of sciatic nerve and spinal cord in control and streptozocin-injected rats

	Sciatic nerve	Spinal cord
Control	114.6 ± 3.4	604.6 ± 12.3
Untreated diabetic	100.0 ± 4.7*†	446.2 ± 11.1†‡
Insulin-treated diabetic	125.5 ± 4.7	570.9 ± 13.1

Values are means ± SE (mg).
*$P < 0.05$, ‡$P < 0.001$, vs. control rats.
†$P < 0.001$ vs. insulin-treated diabetic rats.

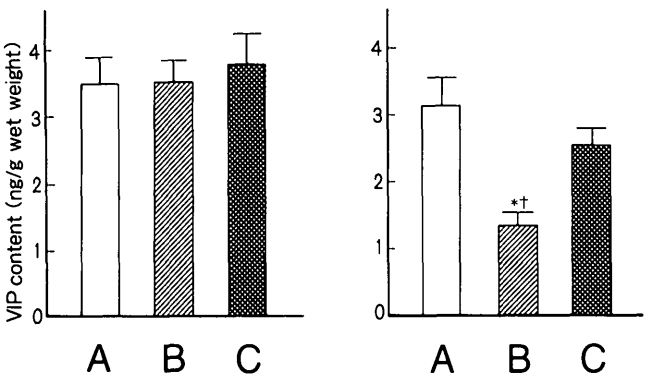

FIG. 3. Vasoactive intestinal polypeptide (VIP) content in spinal cord (*left*) and sciatic nerve (*right*) from control (*A*), untreated streptozocin-induced diabetic (STZ-D) (*B*), and insulin-treated STZ-D (*C*) rats. Values are means ± SE. *$P < 0.01$ vs. controls. †$P < 0.05$ vs. insulin-treated STZ-D rats.

abolic derangement may cause the decrease in VIP content in diabetic peripheral nerve. However, the precise mechanism of this reduction of VIP content in the diabetic sciatic nerve was not clarified in this study.

We observed the increase in caudal NCV in rats aged 10 wk over 8 wk of STZ-D. Indeed, Miyoshi and Goto (22) found that NCV in Wistar rats aged 10 wk increased during growth up to ~23 wk of age. The increase in NCV during this period was ~17 m/s. Gillon et al. (28) also found an increase in NCV of the sciatic nerve during growth. This increase in NCV is presumably due to growth of nerve fibers. Also, in their study, NCV in diabetic rats was slower than in controls. Perhaps this reduction, as suggested by Jakobsen (29), could be explained by some alteration in nerve fibers related to diabetes rather than an inhibition of nerve growth.

Our investigation proves a significant correlation between

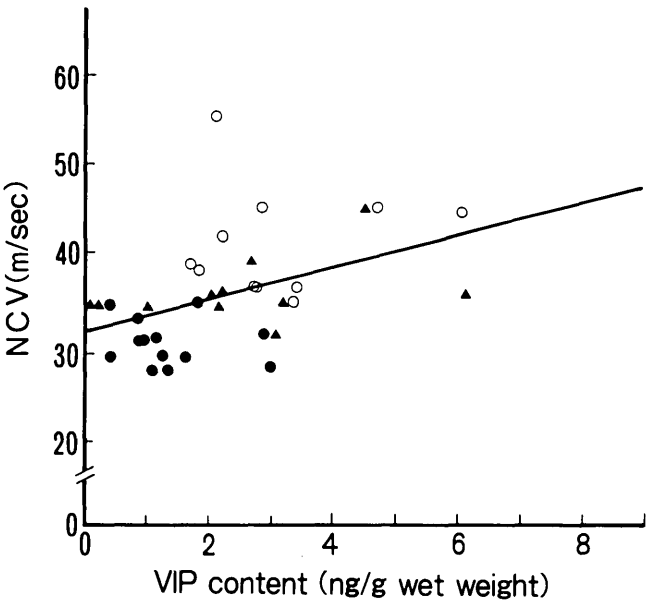

FIG. 4. Relationship between nerve conduction velocity (NCV) and vasoactive intestinal polypeptide (VIP) content in sciatic nerve of experimental rats. ○, Control rats; ●, untreated streptozocin-induced diabetic (STZ-D) rats; ▲, insulin-treated STZ-D rats. $P < 0.01$, $r = 0.430$.

VIP and NCV in nondiabetic and STZ-D rats. Obviously, this evidence does not necessarily prove causal relationship between VIP and neuropathy. However, several lines of circumstantial evidence that suggest an etiologic role for VIP in the development of diabetic neuropathy are present, as discussed below. Interestingly, this study shows that, in diabetic rats, VIP content in sciatic nerve but not spinal cord is decreased. VIP content in spinal cord of STZ-D rats was not different from controls. Previous reports concerning the distribution of VIP immunoreactivity in spinal cord stated that afferent fibers are the main source of VIP in spinal cord, and in particular, the lumbosacral segment receives large numbers of VIP-containing visceral afferents (30–33). The existence of VIP-containing nerve fibers other than visceral afferents in the spinal cord has been confirmed in two independent investigations (34,35). VIP-containing neurons in the spinal cord probably include afferent fibers, supraspinal nerve fibers, and intrinsic spinal neurons, although according to several studies, sciatic nerve presumably includes only two groups of VIP-containing nerve fibers, i.e., sympathetic postganglionic fibers and somatic afferents (36–38). Because of these findings, the decrease of VIP in visceral or somatic afferents cannot explain our observation in STZ-D rats of a loss of VIP immunoreactivity in sciatic nerve but not in spinal cord. Therefore, our observation strongly suggests that the decrease of VIP associated with STZ-D is due to the change of VIP content in the sympathetic postganglionic fibers. Lundberg et al. (36) reported that after lumbosacral sympathectomy, large numbers of VIP-positive fibers around the ligature on sciatic nerve disappeared. This finding suggests that the major population of VIP-containing fibers in the sciatic nerve is sympathetic postganglionic nerve fiber. In general, these sympathetic nerves innervate and regulate the microvascular circulation, including the vasa nervorum of the peripheral nerve. The presence of VIP in nerves in perivascular plexuses of vasa nervorum has been demonstrated by the immunohistochemical study of Appenzeller et al. (20). VIP is a major vasodilative factor in the regulation of blood flow in the gastrointestinal (3) and genital (18) tracts. Therefore, it is reasonable to speculate that a decrease of VIP content in the sympathetic efferent fibers might cause a vasoconstriction of the microvasculature in peripheral nerves, and as a consequence, ischemic damage of the nerve might occur. In fact, Tuck et al. (39) found that disturbance in endoneural blood flow with concomitant insufficient O_2 supply was an important etiologic factor of diabetic peripheral neuropathy.

Although there is little doubt that multiple biochemical abnormalities, including hyperglycemia, polyol metabolism, or other derangements, in diabetes play an etiologic role in the development of diabetic neuropathy (40,41), we speculate that reduced VIP in the autonomic nerve might be another important pathogenetic factor for diabetic peripheral polyneuropathy.

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