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BRIEF COMMUNICATION

Development of Supersensitivity to Substance P in the Spinal Cord of the Streptozotocin-Induced Diabetic Rats

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KAMEI, J., M. OGAWA AND Y. KASUYA. *Development of supersensitivity to substance P in the spinal cord of the streptozotocin-induced diabetic rats.* PHARMACOL BIOCHEM BEHAV **35**(2) 473–475, 1990.—To investigate the possible mechanisms involved in the alterations in sensitivity to pain in diabetic rats, we examined the influence of diabetes induced by streptozotocin (STZ) on the functions of the neuronal systems that contain substance P (SP) within the spinal cord. The threshold for pain perception as determined by a tail-pinch test was significantly reduced in diabetic rats. The levels of SP in the spinal cord from diabetic rats (116.9 ± 16.3 pmol/g tissue) were significantly lower than those from the control rats (190.2 ± 14.1 pmol/g tissue). Diabetic rats were found to have a significant increase in the number of binding sites for SP in dorsal spinal cord. The concentrations of binding sites in diabetic rats and in control rats were 102.1 ± 17.3 fmol/mg protein and 52.6 ± 6.6 fmol/mg protein, respectively. These data indicate that STZ-induced diabetic rats exhibit supersensitivity to SP in the spinal cord. This may be correlated, in part, with the reduction in the threshold for perception of pain in diabetic animals.

Substance P Diabetes Nociception Spinal cord

A recent report by Forman *et al.* (1) indicates that nociceptive transmission is enhanced in experimental models of diabetes. However, little information is available concerning mechanisms responsible for the alterations in nociceptive transmission in the diabetic animals.

The undecapeptide substance P has been proposed as an important neurotransmitter in primary sensory neurons involved in nociception. Substance P is localized in small afferent nerve fibers in the substantia gelatinosa of the dorsal horn in the spinal cord. In addition, substance P is released from slices of spinal cord in vitro upon treatment with high extracellular levels of potassium ions (6,9), and in vivo from spinal cord by application of noxious stimuli (3). Moreover, intrathecal administration of substance P produces irritation and hyperalgesia (5). Since substance P appears to be a nociceptive afferent neurotransmitter, one hypothesis to explain the mechanisms responsible for the alterations in nociceptive transmission in the diabetic animals is that the functions of the neuronal systems that contain substance P within the spinal cord may be altered in diabetic animals. To test this hypothesis, we investigated possible mechanisms involved in the alterations in nociceptive transmission by measuring levels of substance P in the spinal cord and the binding of ^{3}H-substance P to spinal cord membrane from streptozotocin-induced diabetic rats.

METHOD

Male Sprague-Dawley rats (Tokyo Animal Laboratory Inc., Tokyo, Japan), initially 8 weeks old, weighing about 250 g at the beginning of the experiments, were used. Animals were housed in groups of six per cage under a 12-hr light-dark cycle with food and water continuously available. The room temperature was maintained at 22 ± 1°C.

Induction of Streptozotocin-Diabetes

After a one-week adaptation period, animals were rendered diabetic by an injection of streptozotocin (STZ; 60 mg/kg IV) prepared in 0.1 N citrate buffer at pH 4.5. Age-matched control rats were injected with the vehicle alone. The experiments were conducted 8 weeks after injection of vehicle or STZ.

Rats with a serum glucose concentration above 400 mg/dl were considered diabetic.

Measurement of the Threshold for Pain Perception

The threshold for pain perception was measured by tail-pinch method. Hemostatic forceps (2 kg constant pressure) were applied to the root of the tail, and the latency of the biting response to the forceps was measured.

Measurement of Substance P-Like Immunoreactivity (SPLI) in Spinal Cord

Rats were killed by decapitations, the lumbar spinal cord was rapidly removed and stored at −80°C until assay.

The spinal cord tissues were homogenized in 5 volumes (vol./wt.) of 0.1 M HCl and heated for 15 min at 95°C. After centrifugation at 38000 × g for 15 min at 4°C, the supernatant was adjusted to pH 7.0 with 1 M Tris, and the precipitated materials were centrifuged at 8000 × g for 10 min at 4°C. SPLI in the final supernatant was assayed by radioimmunoassay according to the instructions provided by Amersham International Ltd.

Binding of ^{3}H-Substance P to Spinal Cord Membrane

The dorsal halves of the lumbar spinal cord were homogenized in 10 volumes (vol./wt.) of 0.32 M sucrose with a glass-Teflon homogenizer. The homogenate was centrifuged at 1000 × g for 10 min and the resulting supernatant further centrifuged at 17000 × g for 20 min. The pellet, referred to as the crude synaptosomal fraction, was washed three times with 50 mM Tris-HCl buffer and centrifuged at 17000 × g for 20 min. The final pellet was then resuspended in 50 mM Tris-HCl buffer (10 ml/g of initial tissue) that contained 40 μg/ml bacitracin, 4 μg/ml leupeptin, 2 μg/ml chymostatin, 25.4 μg/l thiorphan, 10 mM $MgCl_2$ and 0.02% bovine serum albumin, pH 7.4. The binding assay was performed by a method described by Nakata *et al.* (8), with slight modification. The reaction was initiated by addition of 150 μl of the crude synaptosomal preparation, which corresponded to 250 μg protein, in duplicate, to 0.1–16 nM ^{3}H-substance P at 20°C for 60 min in a final volume of 300 μl which contained the same 50 mM Tris-HCl buffer. At the end of the incubation period, 3 ml of ice-cold buffer were added to each tube and its contents were filtered immediately under reduced pressure through Whatman GF/C glass-fiber filters (pretreated with 0.1% polyethylenamine in water for >3 hr prior to use). Each of the filters was then washed two times with 3 ml of ice-cold buffer and radioactivity was determined by liquid scintillation spectrometry. Nonspecific binding was defined as binding in the presence of 1 μM substance P. Specific binding was calculated by subtracting the nonspecific binding from total binding. Protein was measured by the method of Lowry *et al.* (4).

Drugs

The following drugs were used in this study: streptozotocin was purchased from Sigma Chemical Co., St. Louis, MO. Substance P, with N-succinimidyl-3-(4-hydroxy-5-[^{125}I] iodophenyl) propionate (Bolton and Hunter reagent) for radioimmunoassay and [2-L-prolyl-3,4-^{3}H(N)]-substance P for binding assays for substance P in the spinal cord were purchased from Amersham International, Amersham, Buckinghamshire, U.K. Other reagents were of analytical grade from commercial sources.

Data Analysis

Data are presented as the mean ± S.E. Statistical analyses were performed by analysis of variance, with a Student's *t*-test or Cochran-Cox test.

RESULTS

The body weight of diabetic rats (291.7 ± 11.6 g) was significantly ($p<0.01$) reduced to level 42% below controls (505.1 ± 10.2 g). Serum glucose levels in the diabetic rats (556.6 ± 18.3 mg/dl) were significantly ($p<0.01$) higher than in the controls (152.3 ± 9.0 mg/dl).

TABLE 1

EFFECT OF STZ-INDUCED DIABETES ON LATENCY OF RESPONSE TO TAIL PINCH

	Latency (sec)
Control	14.4 ± 0.8 (n = 35)
Diabetes	6.6 ± 0.8* (n = 21)

Data are represented as mean ± S.E. of results from the number of experiments given in parentheses. *$p<0.01$ compared with control.

Latency of Response to Tail-Pinch Stimuli

Eight weeks after induction of diabetes, the latency of the biting response to forceps was significantly decreased in diabetic rats as compared to controls (Table 1).

Levels of SPLI in the Spinal Cord

The results of experiments designed to investigate the influence of diabetes on levels of SPLI in spinal cord are presented in Table 2. As can be seen from the table, the basal levels of SPLI in the spinal cord of diabetic animals were significantly lower ($p<0.05$) than in controls. The levels of SPLI in the spinal cord of diabetic rats fell by about 40% from the corresponding control values.

Receptors for Substance P in the Spinal Cord

Table 2 also shows the data on binding of ^{3}H-substance P to the membrane of spinal cord from STZ-induced diabetic rats. A significantly higher number of binding sites (B_{max}) was found in the spinal cord of diabetic rats than in controls. In contrast, receptors for substance P in diabetic rats had a relatively low affinity for tritiated ligand. However, no statistically significant change was observed in the apparent K_D for the binding of ^{3}H-substance P to spinal cord.

DISCUSSION

In our study, the threshold for pain perception in the tail-pinch

TABLE 2

SUBSTANCE P CONTENT AND ^{3}H-SUBSTANCE P BINDING IN SPINAL CORD FROM AGE-MATCHED CONTROL AND STZ-INDUCED DIABETIC RATS

	Substance P Content	^{3}H-Substance P Binding	
		B_{max}	K_d
	(pmol/g tissue)	(fmoles/mg protein)	(nM)
Control	190.2 ± 14.1	52.58 ± 6.63	2.96 ± 0.65
Diabetes	116.9 ± 16.3†	102.09 ± 17.26*	6.04 ± 1.59

Data are represented as mean ± S.E. of results from 12 experiments for substance P content and of results from 4 separate experiments for binding data. B_{max} and K_d values were calculated by Scatchard plot analysis of binding data. *$p<0.05$ and †$p<0.01$ compared with control.

test was reduced when animals were tested 8 weeks after induction of diabetes. In addition, specific binding by receptors of ^{3}H-substance P increased significantly in spinal cord 8 weeks after the induction of diabetes. There is evidence that an increased number or density of receptors is responsible for increased sensitivity to neurotransmitters. In spinal cord, Takahashi and Otsuka (11) observed the decrease in endogenous levels of substance P in cat dorsal horn 11–12 days after dorsal-root section. Furthermore, Nakata *et al.* (7) reported that the number of specific binding sites for ^{3}H-substance P in synaptic membrane from rabbit spinal cord increased two or three weeks after dorsal-root section. Thus, when a tissue is denervated and time is allowed for the nerve terminals to degenerate and for the levels of neurotransmitter to decrease, supersensitivity of postsynaptic receptors to the transmitter develops. Indeed, in the present study, rats diabetic for 8 weeks demonstrated a significant decrease in the level of SPLI in the spinal cord. Therefore, the increased number of receptor sites for substance P in diabetic rat spinal cord may have been a response to substantially decreased levels of substance P in tissue. This result suggests that postjunctional supersensitivity to substance P develops in spinal cord of diabetic animals. Furthermore, substance P has been proposed as an excitatory transmitter of primary afferent fibers involved in nociception (3, 5, 6). Thus, it is possible that the development of such supersensitivity to substance P may be the cause of the reduction in the threshold for pain perception.

The mechanisms responsible for decreased levels of SPLI in spinal cord of animals with diabetes of 8 weeks duration is unclear. Reduced rates of protein synthesis in dorsal-root ganglia of diabetic rats have been reported (10), possibly being secondary to reduced uptake of amino acids (12). These findings support the possibility of reduced synthesis of substance P in dorsal-root ganglia of diabetic rats. Reduced synthesis would lead to a reduction in levels of substance P in spinal cord and could explain our finding. However, spinal cord substance P also originates from cells within spinal cord and from cells with descending axons from brainstem. In the present study, we cannot rule out these other sources as contributing to the observed decrease in substance P.

Receptors in diabetic rats had a lower affinity for substance P than those in controls, although this change was not statistically significant. For several neurotransmitter receptors, guanine nucleotides reduced the affinity of agonists (2). If the guanine nucleotides metabolism is altered during diabetic state, the affinity for substance P receptors might be changed. However, we cannot deduce any details of the mechanisms at present, further studies are needed to resolve this problem.

In conclusion, we have shown that STZ-induced diabetic animals exhibited obvious functional changes in the neuronal systems in spinal cord that contain substance P. We found a decrease in substance P contents and an increase in the number of postsynaptic binding sites for substance P in spinal cord. These changes may be correlated, in part, with a reduction in the threshold for pain perception. Whether the functional changes in neurons that contain substance P are the direct result of changes in levels of insulin or blood glucose, or of changes in metabolic parameters not studied, remains to be clarified.

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