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Impairment of neurogenic inflammatory and anti-inflammatory responses in diabetic rats

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Abstract

The effect was studied of a primary (preconditioning) neurogenic inflammatory challenge induced by electrical stimulation of the peripheral stump of the sciatic nerve (20 V, 0.5 ms, 5 Hz, for 5 min) on neurogenic oedema (5 min later) induced by stimulation of the contralateral sciatic nerve. Plasma extravasation due to the second stimulation was decreased by $52.7 \pm 3.1\%$ ($P < 0.01$) in normal animals and by 29.7 ± 2.2 and $18.1 \pm 1.5\%$ with 50 mg/kg streptozotocin pretreatment i.v. 4 and 8 weeks previously, respectively. Subsequently, bilateral sciatic nerve stimulation increased baseline plasma somatostatin levels from 6.4 ± 0.3, 11.7 ± 1.4, and 16.8 ± 3.8 to 28.3 ± 2.9 ($P < 0.01$), 17.9 ± 3.7, and 25.1 ± 1.7 pmol/l in normal, and 4- and 8-week diabetic animals, respectively. We conclude that experimental diabetes impairs the capability of a preconditioning neurogenic inflammatory episode to elicit a systemic anti-inflammatory effect. This is accompanied by a deficiency in elevation of the plasma somatostatin level in response to nerve stimulation, although the baseline plasma somatostatin level increases proportionally to the duration of experimental diabetes.

Keywords: Diabetic rat; Somatostatin; Neurogenic inflammation; Inflammatory preconditioning

1. Introduction

Sensory nerves transmit sensory information and release several biologically active substances (see for reviews Holzer, 1992; Szolcsányi, 1996). Evidence favours a role for calcitonin gene-related peptide (CGRP), tachykinins such as substance P and neurokinin A, nitric oxide and somatostatin (see for reviews Moncada and Higgs, 1995; Lundberg, 1996; Szolcsányi, 1996). These transmitters released from sensory nerve terminals have been described to mediate vasoactive effects predominantly at the microvascular level, thus contributing to the regulation of blood flow in peripheral tissues and to ongoing inflammatory and wound healing processes (see for review Brain, 1996). Somatostatin of sensory neural origin and resulting from local neurogenic inflammation has been found to mediate systemic anti-inflammatory effects to produce hormone-like anti-inflammatory activity in rats (Pintér and Szolcsányi, 1996; Szolcsányi et al., 1998a,b).

Sensory neural dysfunction commonly occurs in patients with peripheral neuropathy, a major complication of diabetes mellitus. Insulin-sensitive depletion of sensory neuropeptides such as substance P, CGRP and somatostatin is characteristic of experimental insulin-deficient diabetes mellitus (Gamse and Jancso, 1985; Diemel et al., 1992; Nemeth et al., 1999) and has been suspected to underlie an attenuated neurogenic inflammatory response in both experimental animals and diabetic patients (Walmsley and Wiles, 1991; Gyorfi et al., 1996). Together, the key role of somatostatin in the development of the systemic anti-inflammatory effect provoked by a primary neurogenic inflammatory episode (Szolcsányi et al., 1998a,b) and a decreased somatostatin release in response

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to a standardized challenge in experimental diabetes (Nemeth et al., 1999), have led us to investigate the possibility that the somatostatin-mediated systemic anti-inflammatory adaptive mechanism may be impaired in the insulin-deficient diabetic state in rats.

2. Methods

2.1. Ethics

The experiments performed in the present work conformed to the European Community guiding principles for the care and use of laboratory animals. In addition, the experimental protocol applied was approved by the local ethical committee of the Medical University of Pécs, Hungary.

2.2. Experimental groups

The experiments were carried out with 72 male Wistars rats weighing 200–230 g. The animals were kept in the Laboratory Animal Center of the University Medical School of Pécs under pathogen-free conditions (12-h light/dark periods a day, temperature of 22–25°C, humidity of 50–70%) and were provided with standard rat chow and tap water ad libitum. The rats were randomized into three experimental groups. Group 1: animals without streptozotocin, Groups 2 and 3: animals treated with 50 mg/kg streptozotocin i.v. 4 or 8 weeks prior to the experiments, respectively. Each group consisted of 24 rats; six animals for determination of plasma extravasation, 12 for the measurement of fasting plasma somatostatin, insulin and blood glucose levels and six for nerve conduction velocity tests.

2.3. Induction of neurogenic inflammation

Neurogenic inflammation was induced by electrical nerve stimulation as follows: the rats were anaesthetized with sodium thiopentone (50 mg/kg i.p.). The left jugular vein was cannulated, and a T-tracheal tube connected to a small animal respirator (KUTESZ, Budapest, Hungary) was inserted for artificial respiration as described (Pintér et al., 1997). The sciatic nerves were exposed and cut in the thigh and the surrounding skin flaps were fixed to a metal ring to make a pool filled with liquid paraffin. The peripheral stumps of the nerves were placed on pairs of platinum hook electrodes and stimulated with C-fibre strength (20 V, 0.5 ms, 5 Hz, 5 min), (Pintér and Szolcsányi, 1996; Szolcsányi et al., 1998a). Guanethidine (8 mg/kg, i.p.) was given 1 h before nerve stimulation to counteract the vascular effects of concomitant sympathetic nerve excitation (Pintér et al., 1997). Pipecuronium bromide (200 μg/kg, i.v.) was injected to block neuromuscular transmission. Stimulation of the right sciatic nerve was followed by stimulation of the left one (or vice versa) with an interval of 5 min between the first and second stimulation periods. Plasma extravasation was determined with the Evans blue accumulation method.

2.4. Plasma extravasation

Evans blue dye (50 mg/kg, i.v.) was given 30 min after acute denervation. The animals were exsanguinated 20 min after the second sciatic nerve stimulation. The cutaneous areas supplied by the sciatic nerve (plantar and lateral dorsal skin of the hindpaw) were cut out. The dye content of the tissue samples was extracted with formamide over 72 h at room temperature for photometric determination at 620 nm (Spectromom 195, MOM, Budapest, Hungary). Evans blue accumulation was expressed as μg dye/g wet tissue weight as described (Pintér and Szolcsányi, 1996; Szolcsányi et al., 1998a,b).

2.5. Protocol for blood sampling

In addition to the instrumentation described above (Section 2.4), a polyethylene cannula was inserted into the right carotid artery to collect blood. Bilateral sciatic nerve stimulation was performed (20 V, 0.5 ms, 5 Hz, 5 min) 2 min preceding blood sampling for plasma somatostatin measurement. For control, samples were also taken from rats whose sciatic nerves were cut but not stimulated. These experiments were carried out with animals that had been fasted for a 12-h period.

2.6. Determination of plasma somatostatin, insulin and blood glucose concentrations

Arterial blood samples (3 ml/rat) were taken into ice-cold tubes containing EDTA (6 mg) and Trasylol (1000 IU) 2 min after discontinuation of the stimulation. The samples were then centrifuged at 4°C (2000 rpm, for 10 min), the somatostatin content of 1 ml plasma was extracted by addition of 3 vol. of absolute alcohol. After precipitation and a second centrifugation with the same parameters, the supernatants were aspirated off then evaporated under nitrogen. Plasma somatostatin immunoreactivity was determined by radioimmunoassay (RIA) as described (Nemeth et al., 1996, Szolcsányi et al., 1998a,b). Plasma insulin levels were measured by RIA, and blood glucose levels with the glucose oxidase–peroxidase method. Of course, plasma insulin and blood glucose levels were also measured in animals without nerve stimulation.

2.7. Nerve conduction velocity

Left femoral nerve conduction velocity was determined in subgroups of normal (Group 1) and diabetic animals

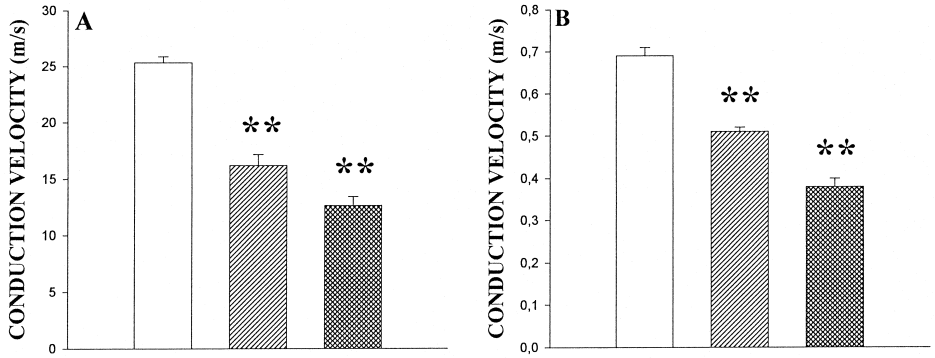

Fig. 1. Femoral nerve conduction velocity values for A- (A) and C-fibres (B) in normal and diabetic rats. Blank columns: non-diabetic controls (Group 1); hatched columns: 4-week streptozotocin-diabetic animals (Group 2); cross-hatched bars: 8-week diabetic animals. The data are expressed as means obtained with six animals in each group. Error bars are S.E.M. Significantly different from Group 1 at $^{*}P < 0.05$ and $^{**}P < 0.01$.

(Group 2 and 3). In thiopentone-anaesthetized animals (Section 2.3), the nerve was prepared, cleaned of fat and adhering connective tissue and trains of square-wave constant voltage stimuli were applied through a pair of platinum electrodes placed as high as possible. The intensity, frequency and number of stimuli varied but the pulse width (500 μs) was kept constant. Another pair of electrodes was applied approximately 2 cm distal to the stimulating electrodes for recording the summation action potentials evoked by the proximal stimulation. The time lags between stimulation and the appearance of corresponding ''A'' and ''C'' waves were determined, reflecting activation of populations of A- and C-fibres, respectively. Average conduction velocity (m/s) was calculated by dividing the distance between the stimulating and receiving electrodes by the interval between the end of the stimulatory impulses (20 stimuli) and the appearance of the corresponding ''A'' and ''C'' signals (Janig and Lisney, 1989).

2.8. Drugs and solutions

Sodium thiopentone (Trapanal) was purchased from Byk Gulden (Konstanz, Germany), streptozotocin (Zanosar) from Upjohn (Kalamazoo, USA), guanethidine, somatostatin-14, [Tyr^{1}]somatostatin-14 from Sigma (St. Louis, USA), pipecuronium bromide (Arduan), trasylol (Gordox) from Richter (Budapest, Hungary), EDTA, formamide, Evans blue dye from Reanal (Budapest, Hungary), insulin RIA kit from Izinta (Budapest, Hungary). ^{125}I-labelled somatostatin RIA tracer was prepared in our laboratory.

2.9. Statistical analysis

The data are expressed as means ± standard error of means (S.E.M.). Plasma extravasation values were evaluated with a non-parametric Mann–Whitney test, plasma somatostatin, insulin, blood glucose concentrations and sciatic nerve conduction velocity values were analyzed with Student's *t*-test for unpaired data. Changes were considered significant at $P < 0.05$.

3. Results

3.1. Effects of diabetes on body weight, fasting blood glucose and plasma insulin levels

The control (Group 1) animals grew steadily over the 8-week experimental period with an average weight gain of 72 ± 4.8 g, whereas animals in Group 2 and Group 3 had a weight loss of 6.3 ± 1.1 and 10.7 ± 2.4 g, respectively.

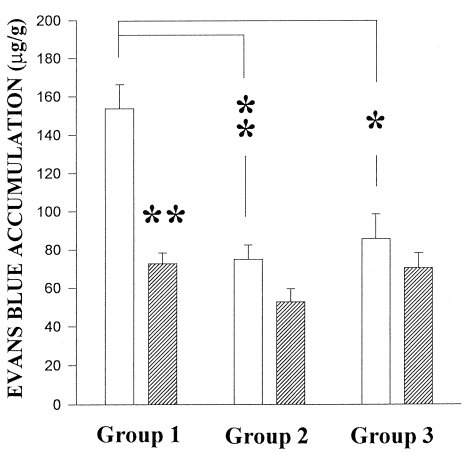

Fig. 2. Plasma extravasation evoked in the skin of the hindpaw by two consecutive periods (5-min interval) of stimulation (20 V, 0.5 ms, 5 Hz, 5 min) of the peripheral stump of the sciatic nerve in normal and diabetic rats. Group 1: non-diabetic control; Group 2: 4-week, and Group 3: 8-week diabetic rats. Blank columns represent plasma extravasation provoked by the 1st (preconditioning) stimulation, hatched bars show values due to the 2nd stimulation on the other side. The data are expressed as means obtained with six animals in each group. Error bars are S.E.M. Significant difference between 1st and 2nd stimulation values within groups at $^{*}P < 0.05$ and at $^{**}P < 0.01$, otherwise as indicated.

Fasting blood glucose levels were 6.0 ± 0.3 (Group 1), 23.6 ± 3.1 (Group 2; $P < 0.01$), and 18.4 ± 3.2 mmol/l (Group 3; $P < 0.01$). Plasma insulin concentrations were significantly decreased from 10.5 ± 2.7 (Group 1), to 2.5 ± 0.6 (Group 2; $P < 0.05$), and 2.8 ± 0.9 μIU/ml (Group 3; $P < 0.05$).

3.2. Nerve conduction velocity test: evidence for the presence of diabetic neuropathy

Fig. 1 shows the diabetes-induced decrease in nerve conduction velocity in fast conducting myelinated (A-fibres in Fig. 1A) and slow conducting unmyelinated (C-fibres in Fig. 1B) fibres. At a stimulation intensity suprathreshold for A- (0.5 V, 5 Hz) or C- (3 V, 5 Hz) fibres, conduction velocity decreased progressively with the duration of the pre-existing diabetic state.

3.3. Effect of diabetes on cutaneous neurogenic inflammation and the systemic anti-inflammatory effect evoked by antidromic sciatic nerve stimulation

The distal stump of the right sciatic nerve was stimulated with C-fibre strength (1500 pulses), for 5 min followed by the same stimulation on the contralateral side. Evans blue accumulation in the skin of both hindpaws was then determined. In Group 1, the primary neurogenic inflammation resulted in $52.7 \pm 3.1\%$ inhibition of the secondary neurogenic cutaneous plasma extravasation of the contralateral hindleg ($P < 0.01$). Diabetes was found to produce ''per se'' an inhibitory effect on the primary inflammatory response characterized by a significant decrease in plasma extravasation as compared to that seen in Group 1. In Groups 2 and 3, the plasma extravasation produced by the 1st and 2nd stimulations did not differ from each other (Fig. 2).

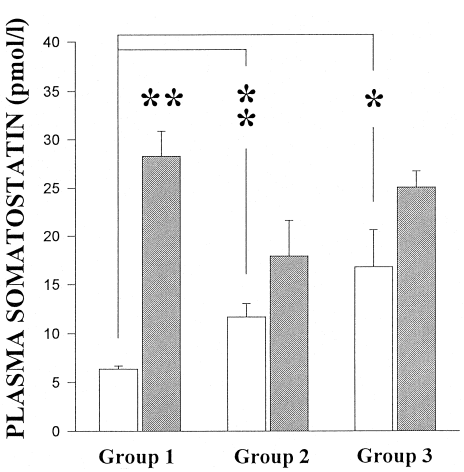

Fig. 3. Plasma somatostatin levels in normal and diabetic rats. Group 1: non-diabetic control; Group 2: 4-week, and Group 3: 8-week diabetic rats. Blank columns represent baseline levels, hatched columns represent values obtained after a single period of bilateral sciatic nerve stimulation (20 V, 0.5 ms, 5 Hz, 5 min). The data are expressed as means obtained with six animals in each group. Error bars are S.E.M. Significant difference between baseline and post-stimulation values within groups at $^{*}P < 0.05$ and $^{**}P < 0.01$, otherwise as indicated.

3.4. Effects of diabetes mellitus on the level of plasma somatostatin — changes in response to bilateral antidromic sciatic nerve stimulation

Bilateral sciatic nerve stimulation elicited an approximately 4-fold increase in plasma somatostatin concentration in Group 1 animals. Baseline plasma somatostatin levels were significantly increased by diabetes of either 4 weeks (Group 2) or 8 weeks (Group 3) duration. However, bilateral nerve stimulation failed to increase the plasma somatostatin level in Groups 2 and 3 (Fig. 3).

4. Discussion

The results confirmed previous findings that the plasma somatostatin level increases in insulin-deficient experimental diabetes (Fischer et al., 1998; Nemeth et al., 1999) and that streptozotocin diabetes attenuates the primary neurogenic inflammatory response characterized by plasma extravasation in streptozotocin diabetic rats (Gamse and Jancso, 1985; Gyorfi et al., 1996). The results also show that sensory nerve stimulation results in a dramatic increase in plasma somatostatin levels with the development of a systemic anti-inflammatory effect indicated by significant alleviation of plasma extravasation in response to a standardized inflammatory challenge in otherwise healthy rats (Szolcsányi et al., 1998a,b). The lack of a difference between amplitudes of plasma extravasation in response to repetitive inflammatory insults in insulin-deficient experimental diabetes is the major original finding of the present work i.e., the *inflammatory preconditioning* phenomenon is lost in diabetes.

The streptozotocin-treated rats exhibited characteristic features of Type I (insulin-dependent) diabetes in that they failed to gain weight, suffered from hyperglycaemia, and on direct determination of fasting plasma insulin levels, showed a substantial insulin deficiency. Moreover, the nerve conduction velocity test, the gold standard for verifying diabetic neuropathy (Love et al., 1996; Cameron and Cotter, 1997; Kato et al., 1998) confirmed that the diabetic animals suffered from a superimposing sensory neuropathy.

According to the report of the San Antonio Conference (1988), ''diabetic neuropathy is a descriptive term meaning a demonstrable disorder, either clinically evident or subclinical, that occurs in the setting of diabetes mellitus without other causes for peripheral neuropathy''. As an experimental approach, streptozotocin-induced diabetes has been extensively used to study the pathogenesis of diabetic neuropathy. In this model, neuropathy, similar to that seen

in the Type I diabetes typically involves detrimental changes in autonomic, sensory and motor nerves (Soediono et al., 1993; Love et al., 1996; Kato et al., 1998). As far as the pathomechanism of sensory neuropathy associated with Type I diabetes is concerned, a defective axonal transport including that of sensory neuropeptides is believed to be a critical initiating factor in degenerative distal neuropathies leading to severe microcirculatory changes in both diabetic patients and experimental animals (Ralevic et al., 1993; see for review, Fedele and Giugliano, 1997; Bennett et al., 1998). As the local effector function of peripheral sensory nerves is known to be underlain by the ability of these nerves to release neuropeptides in response to various stimuli (see for reviews Brain, 1996; Szolcsányi, 1996), it is not surprising that streptozotocin diabetes, a state characterized by a deficient sensory neuropeptide content/release has a profound influence on neurogenic inflammation. Depletion of CGRP and substance P content has been shown to occur in sensory nerves of streptozotocin-diabetic rats (Diemel et al., 1992), and direct measurement of CGRP, substance P and somatostatin released in response to a highly standardized challenge evidenced a parallel attenuation of the release of these sensory neuropeptides from isolated trachea of the rat with a pre-existing 4-week streptozotocin diabetes (Nemeth et al., 1999).

Beyond its local effector function, somatostatin has recently been shown to underlie a systemic anti-inflammatory effect associated with neurogenic inflammation (Szolcsányi et al., 1998a,b). This means that a preceding neurogenic inflammatory episode significantly decreases the intensity of a subsequent one induced by either electrical stimulation or counter-irritants even at remote sites. It has also been proven that the protective effect is mediated by circulating somatostatin predominantly of sensory neural origin (Szolcsányi et al., 1998a,b). The phenomenon resembles that of preconditioning, a term originally coined by Murry et al. (1986) for the dramatically increased tolerance of the myocardium to a normally lethal ischaemic injury achieved by an initial brief exposure to ischaemia. Later, Kloner's group has shown that transient ischaemia of the gastrocnemius muscle combined with electrical stimulation significantly decreased infarct size due to prolonged coronary artery occlusion in rabbits (Birnbaum et al., 1997). Moreover, Ferdinandy et al. (1997a) elegantly demonstrated that the preconditioning phenomenon depended on the functional integrity of capsaicin-sensitive sensory nerve fibres. Therefore, we think that, analogous to the ischaemic preconditioning phenomenon, the ability of a preceding neurogenic inflammation to reduce the intensity of a succeeding one can be termed *inflammatory preconditioning*. Myocardial preconditioning, however, seems to be a healthy-heart phenomenon since both hyperlipidaemia and streptozotocin diabetes block the phenomenon (Szilvassy et al., 1995; Tosaki et al., 1996; Ferdinandy et al., 1997b; see for review Ferdinandy et al., 1998). The present results also show that the *inflammatory preconditioning* phenomenon was also blocked by streptozotocin diabetes. As indicated by the results presented in Fig. 2, plasma extravasation produced by the primary neurogenic inflammatory episode was significantly decreased in diabetic animals and this was accompanied by a significantly increased baseline plasma somatostatin level (Fig.3). However, the electrical stimulation protocol applied failed to further increase plasma somatostatin concentrations, and there was no *inflammatory preconditioning* phenomenon. This is consistent with the concept that it is neural somatostatin which is of crucial importance in triggering the development of the inflammatory adaptive mechanisms; nevertheless, an increase in plasma somatostatin of metabolic origin can also mediate an anti-inflammatory effect.

In summary, streptozotocin-induced diabetes is referred to as a condition which renders the organism relatively resistant to interventions that provoke neurogenic inflammation. On the other hand, possibly due to a deficient neural somatostatin release mechanism, insulin-deficient diabetes blocks the *inflammatory preconditioning* phenomenon in rats.

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