Feeble bronchomotor responses in diabetic rats in association with decreased sensory neuropeptide release

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—Type I diabetes is associated with a low incidence of asthma. We tested whether a decrease in sensory neuropeptide release is associated with an attenuated bronchoconstrictive response to field stimulation (FS; 100 stimuli, 20 V, 0.1 ms, 20 Hz) in streptozotocin (STZ)-induced diabetes. The organ fluid of the preparations were also tested for substance P, calcitonin gene-related peptide (CGRP), and somatostatin concentrations by RIA. Preparations were from either normal rats or those pretreated with 50 mg/kg STZ iv 8 wk before experiment. A group of STZ-treated animals was supplied with insulin delivery (4 IU/day sc) implants between 4 and 8 wk. A subgroup was formed to study the effect of capsaicin desensitization. The atropine-resistant contraction was attenuated by diabetes without capsaicin-sensitive relaxation response. Exogenous CGRP and substance P potentiated, whereas somatostatin inhibited (1 nM–10 μM) the FS-induced contractions in rings from either group. FS released somatostatin, CGRP, and substance P from 0.17 ± 0.024, 0.15 ± 0.022, and 1.65 ± 0.093 to 0.58 ± 0.032, 0.74 ± 0.122, and 5.34 ± 0.295 in preparations from normal, and from 0.19 ± 0.016, 0.11 ± 0.019, and 0.98 ± 0.116 to 0.22 ± 0.076, 0.34 ± 0.099, and 1.84 ± 0.316 fmol/mg wet wt in preparations from diabetic rats. Insulin supplementation restored neuropeptide release in rings from STZ-treated rats. The results show that the decreased FS-induced contractions occurred with a decrease in sensory neuropeptide release in STZ-diabetic rats.

Furthermore, some forms of hyperreactivity can be prevented by sectioning of the vagus nerve (26). Considering that almost 90% of the vagal nerve comprises sensory fibers, it is not surprising that capsaicin pre-treatment can prevent some forms of airway hyperreactivity (22). Alternatively, infusion of sensory neuropeptides induces hyperreactivity in guinea pigs (8).

We have found that the release of sensory neuropeptides, such as that of calcitonin gene-related peptide (CGRP), substance P, and somatostatin, is significantly decreased from isolated tracheae of rats with diabetic sensory neuropathy (20). Given this decrease in sensory neuropeptide release together with the well-documented attenuation of contractile responses of tracheal preparations from insulin-deficient rats to field stimulation (FS) in other studies, we sought to find whether there would be an association between the two processes in the same set of experiments. Here we show that attenuation of field stimulation-induced contractions of the bronchial rings from diabetic rats closely relates to a deficient sensory neuropeptide release.

METHODS

Ethics. The experiments performed in the present work conform to European Community guiding principles for the care and use of laboratory animals. The experimental protocol applied has been approved by the local ethical boards of the Medical Universities of Pécs and Debrecen, Hungary.

Experimental groups. The study was carried out with 48 male Wistar rats weighing 200–210 g and 12 male Dunkin-Hartley guinea pigs (400–420 g). They were housed in an animal room (12-h light/dark periods a day, temperature of 22–25°C, humidity of 50–70%) with four animals per pen and fed commercial laboratory chow and tap water ad libitum. The animals were randomly divided into two experimental groups. The control animals were treated with the solvent for streptozotocin (STZ), whereas the rats in the second group were treated with 50 mg/kg STZ iv (Zanosar; Upjohn, Kalamazoo, MI) to make them diabetic. After 4 wk, the STZ-treated animals were further randomized into two additional groups, one of which comprised animals that were

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supplied with continuous-delivery (4 IU/day) subcutaneous insulin implants (Linplant, Mollegaard, Ejby, Denmark). This group was referred to as the insulin-supplemented group. The implants were placed at the back of the neck under Trapanal anesthesia (45 mg/kg ip). The other subgroup of rats received matching placebo implants (diabetic group). From the diabetic and the insulin-supplemented groups of animals, we formed subgroups to study the effect of pretreatment with neurotoxic capsaicin doses on FS-induced bronchomotor responses and neurotransmitter release studies (n = 6 animals per subgroups)

Isometric tension measurements. Isolated segments of the main bronchi (2 mm) were mounted horizontally on two small L-shaped glass hooks, one of which was connected to a force transducer for measurement of isometric tension. The experiments were carried out in thermostatically controlled (37 ± 0.2°C) organ bath (5 ml) (TSZ 02, Experimetria UK, London, UK) containing Krebs solution. The organ fluid was gassed with 95% O2 and 5% CO2 to maintain pH at 7.40 ± 0.05. Neural effects on contractile activity of the segments were elicited by means of FS (100 stimuli at 20 V, 0.1 ms, and 20 Hz at an initial tension of 12 mN). The rings were prepared from six animals in each group. To study whether the FS protocol applied was selective for nerve-mediated responses, we preincubated some rings for a period of 10 min with tetrodotoxin (TTX), a fast sodium channel blocker.

Neurotransmitter release studies. These have been described in detail elsewhere (20). In brief, after the animals were killed by exsanguination, the lower third of the tracheae with the main bronchi were removed and cleaned of fat and adhering connective tissues. They were prepared for perfusion in a temperature (37°C)- and pH (7.2)-controlled Krebs solution. The organ fluid was gassed with 95% O2 and 5% CO2 to maintain pH at 7.40 ± 0.05. Neural effects on contractile activity of the segments were elicited by means of FS (100 stimuli at 20 V, 0.1 ms, and 20 Hz at an initial tension of 12 mN). The rings were prepared from six animals in each group. To study whether the FS protocol applied was selective for nerve-mediated responses, we preincubated some rings for a period of 10 min with tetrodotoxin (TTX), a fast sodium channel blocker.

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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 Triton X-100 (1,000 IU). The samples were then centrifuged at 4°C (2,000 rpm for 10 min). The somatostatin content of 1 ml plasma was extracted by addition of three volumes of absolute ethanol. After precipitation and a second centrifugation with the same parameters, the supernatants were aspirated and subsequently evaporated under nitrogen as described (9). Plasma somatostatin immunoreactivity was determined by means of RIA (19, 30, 31). Plasma insulin and blood glucose levels were measured by RIA and the glucose peroxidase method, respectively.

Nerve conduction velocity studies. This series of experiments was carried out to verify or exclude diabetic sensory neuropathy. Left saphenous nerve conduction velocity was determined in subgroups of normal, diabetic, and STZ plus insulin-treated animals. In artificially ventilated animals anesthetized with thiopental sodium (50 mg/kg ip), the nerve was prepared and cleaned of fat and adhering connective tissues, and strains of square-wave (500 μs) constant-voltage stimuli were applied through pairs of platinum electrodes placed as high as possible. Another pair of electrodes was applied 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 signals were determined for calculation of average conduction velocity by dividing the interelectrode distance by the interval between the end of the stimulatory impulse and the appearance of the A and C signals (14).

Treatment with capsaicin. Capsaicin was used to elicit a selective functional deterioration of a significant portion of sensory C fibers. Rats constituting subgroups from normal and diabetic animals (n = 6 for each) were given capsaicin/solvent subcutaneously in the sequence of 10, 30, and 50 mg/kg single daily doses over 3 days on the 8th wk of the experimental period. Capsaicin (1% wt/vol) was dissolved in physiological saline containing 3% vol/vol ethanol and 4% vol/vol Tween 80. The animals pretreated with capsaicin were used for further studies after a 3-day period of recovery to avoid nonspecific effects of systemic capsaicin administration as described previously (10).

Mechanical responses to ovalbumin in isolated tracheae from sensitized normal and diabetic guinea pigs. Twelve male Dunkin-Hartley guinea pigs (400–420 g) were randomized into two groups. The control animals were treated with the solvent for STZ, whereas the second group of animals was treated with a single intraperitoneal injection of 180 mg/kg STZ (Zanosar). Four weeks after STZ and/or solvent injection, the animals were actively sensitized by two intraperitoneal injections of 1 ml/kg 5% (wt/vol) ovalbumin (grade III; Sigma, St. Louis, MO) on two consecutive days. The animals were killed after an additional period of 4 wk for isolated trachea experiments. The trachea was cut into single rings that were tied together forming up to six four- to five-ring chains and suspended in 15-ml organ baths containing temperature (37°C)- and pH (7.2)-controlled Krebs buffer continuously aerated with carbogen. The ends of the chains were tied at the bottom of the tissue bath and connected to a force transducer for measurement of isometric tension (TSZ 03, Experimetria UK). The initial tension was set at 15 mN, and after an equilibration period of 60 min the chains were exposed to cumulative increases in ovalbumin concentration (10–11–10–7 g/ml organ bath volume). When the maximum contraction to ovalbumin had been reached, 3 mM carbachol was additionally applied to define the maximum contraction of each tracheal chain. The contractile responses to ovalbumin were expressed as percentage values of the carbachol-induced maximum responses. Only one concentration-response curve of ovalbumin was generated with each chain.

Drugs and solutions. Thiopental sodium (Trapanal, EGIS, Budapest, Hungary) was purchased by Byk Gulden (Konstanz, Germany), STZ (Zanosar) from Upjohn, guanethidine, atropine, somatostatin, CGRP, substance P, and TTX from Sigma, and capsaicin from Fluka (Buchs, Switzerland). Trasylol was from Richter (Budapest, Hungary), and insulin RIA kits were from Izinta (Budapest, Hungary). 125I-labeled RIA tracers were prepared in our laboratory.

Experimental protocol. Eight weeks after treatment with STZ or solvent, the animals were either exsanguinated for in vitro experiments and laboratory determinations or used for nerve conduction velocity studies. Food was withdrawn 12 h before blood sampling for glucose, plasma insulin, and somatostatin measurements. Insulin and somatostatin immunoreactivity were determined by means of RIA (19, 30, 31). The lower third of the tracheae with the main bronchi was then isolated for isometric tension measurements and neurotransmitter release studies. Six separate animals per group entered the nerve conduction velocity study group.

Statistical analysis. The isometric tension and nerve conduction velocity data expressed as means ± SD were evaluated with analysis of variance followed by a modified t-test according to Bonferroni’s method (32). The blood chemistry...
data and sensory neuropeptide levels were evaluated by Student’s t-test for unpaired data.

RESULTS

Effects of experimental diabetes on body weight, blood glucose, plasma insulin, and somatostatin levels. The normal animals grew steadily over the 8-wk observation period with an average weight gain of 62 ± 4.1 and 58 ± 6.1 g, respectively. The diabetic animals exhibited a marginal weight loss (5.0 ± 2.1 g). The insulin-supplemented rats failed to grow during the first 4 wk. Insulin supplementation from the slow release implants (~4 IU/day) during wk 4–8 caused a significant increase in body weight to a level approaching that seen in normal animals (Fig. 1). In normal, diabetic, and insulin-supplemented animals, fasting blood glucose levels were 4.4 ± 0.6, 17.4 ± 5.5, and 5.0 ± 0.6 mmol/l (P < 0.001 between diabetic vs. normal or insulin supplemented), with plasma insulin levels of 11.4 ± 3.2, 2.0 ± 0.4 (P < 0.001 vs. normal), and 12.9 ± 3.8 μIU/ml, respectively. Fasting plasma somatostatin level significantly increased in diabetic vs. normal animals. In response to insulin supplementation, plasma somatostatin level renormalized by the end of the 8-wk period (Fig. 2). Sampling for these determinations was done at the end of the 8-wk experimental period.

The guinea pigs receiving the solvent for STZ exhibited a weight gain of 45 ± 6.4 g over the 8-wk observation period, whereas body weight of the STZ-treated animals did not show any change.

Nerve conduction velocity. Figure 3 shows the diabetes-induced decrease in nerve conduction velocity in fast-conducting myelinated (A fibers in Fig. 3A) and slow-conducting unmyelinated (C fibers in Fig. 3B) fibers. At a stimulation intensity suprathreshold for A (0.5 V, 5 Hz) or C (3 V, 5 Hz) fibers, conduction velocity significantly decreased in diabetic rats. In the insulin-supplemented animals, conduction velocity for either A or C fibers did not differ from those determined in the control group.

Contractile responses to FS. Preparations from normal animals exhibited a biphasic response to FS, i.e., an initial contraction was followed by relaxation (Figs. 4 and 5A). The rings from diabetic rats responded with attenuated monophasic contractions to FS compared with those seen in preparations from normal or insulin-supplemented animals (Figs. 4 and 5B). FS failed to induce any change in tension in rings preincubated with TTX.

In rings from normal rats, both atropine (1 μM) and capsaicin desensitization significantly decreased contractions produced by FS (Fig. 5A). In addition, an augmented relaxation response was seen after atropine, whereas pretreatment with capsaicin abolished the relaxation response to FS (Fig. 5A). In preparations from diabetic animals, capsaicin failed to significantly influence contractions by FS. The inhibitory effect of atropine on FS-induced contractions was striking. Atropine revealed a weak FS-induced relaxation response in preparations from diabetic animals (Fig. 5B). Preparations from the insulin-supplemented animals exhibited essentially similar responses to those seen in preparations from normal rats.

Sensory neuropeptide release. FS-induced release of somatostatin, CGRP, and substance P was significantly attenuated in preparations from STZ-treated rats than in those from normal animals (Fig. 6). Insulin supplementation yielded complete restoration of FS-induced sensory neuropeptide release in STZ-treated rats. FS failed to elicit any significant neuropeptide release from preparations obtained from subgroups of rats that underwent pretreatment with capsaicin. Similarly, no neuropeptide release was seen with preparations from either main group preincubated with 1 μM TTX (Fig. 6).
Effect of sensory neuropeptides on FS-induced contractions. Somatostatin and CGRP were without effect on isometric tension in mechanically precontracted rings in the absence of FS in preparations from either normal or diabetic animals. Substance P, however, produced a concentration-dependent increase in tension with maximum contraction of 12.3 ± 2.7 and 13.6 ± 3.4 mN with \( \log EC_{50} \) of 7.1 ± 0.2 and 7.0 ± 0.1 in preparations from normal and diabetic animals, respectively. Therefore, when the effect of substance P on FS-induced contractions was studied, the initial tension was reset each time to maintain a 12-mN resting tension before an FS challenge.

CGRP (up to 0.1 \( \mu \)M) and substance P (up to 1 \( \mu \)M) augmented the contractile response to FS in rings from both normal and diabetic rats. The potentiating effect of either neuropeptide on FS-induced increase in tension was significantly elevated in preparations from diabetic vs. normal animals. Somatostatin decreased contractions by FS in both normal and diabetic preparations with a significantly attenuated inhibitory effect in bronchial rings from diabetic animals (Fig. 7).

Antigen-induced trachea contraction. In tracheal chains from nondiabetic ovalbumin-sensitized guinea pigs, cumulative increases in ovalbumin concentration in one-log unit steps produced concentration-dependent contractions with maximum values ~70% of those attained by 1 mM carbachol. The concentration-response curve for ovalbumin, however, was shifted to the right when the tracheal chains were prepared from diabetic animals (Fig. 8). The \( EC_{50} \) values for ovalbumin-induced contractions were \( 4 \times 10^{-10} \) and \( 6 \times 10^{-9} \) g/ml in chains from normal and diabetic animals, respectively. The maximum contractions by ovalbumin were also significantly decreased in preparations from diabetic animals (Fig. 8).

DISCUSSION

These results show that STZ diabetes of 8-wk duration attenuates FS-induced bronchoconstriction/relaxation in vitro in rats and decreased bronchial contractions in response to antigen challenge in ovalbumin-sensitized guinea pigs. This attenuated bronchomotor response to antigenic stimuli suggests a potential role for diabetes in the modulation of bronchial reactivity.

**Fig. 3.** Diabetes-induced decrease in nerve conduction velocity in fast-conducting myelinated (A) and slow-conducting unmyelinated (B) fibers of the femoral nerve. Measurements were accomplished 8 wk after a single 50 mg/kg iv dose of STZ. The data are means ± SD obtained with 6 animals/group. #Significantly different from normal and STZ÷insulin at \( P < 0.05 \).

**Fig. 4.** Original tracings representing changes in isometric tension (mN) in bronchial preparations from normal and diabetic rats in response to field stimulation (20 V, 0.1 ms, 20 Hz, 100 stimuli). The effect of atropine and capsaicin. Atropine (1 \( \mu \)M) was added directly to the organ bath, whereas capsaicin was applied as a sequential systemic pretreatment schedule to destroy the capsaicin-sensitive population of C fibers (see METHODS). A: untreated (control); B: after atropine; C: after capsaicin. Top: bronchial rings from normal animals. Bottom: rings from diabetic animals.
response occurs in parallel with a significant decrease in the release of three sensory neuropeptides, such as that of somatostatin, substance P, and CGRP, in response to a highly standardized FS challenge in rats, which is the major original finding of the paper. The FS-induced bronchomotor response and the neuropeptide release were blocked by TTX, a fast Na⁺ channel blocker; thus both can be considered to be of neural origin. These decreased responses were accompanied by a decline of femoral nerve conduction velocity in the STZ-treated animals. Because the nerve conduction velocity test is widely accepted as the “gold standard” of diabetic neuropathy (6, 15, 17), it is also confirmed that the diabetic animals suffered from sensory neuropathy 8 wk after STZ injection. Alternatively, because insulin supplementation restored both the deficient sensory neuropeptide release with reduced bronchomotor responses and femoral nerve conduction velocity abnormalities in STZ-treated animals, these alterations were considered to result from uncontrolled diabetes.

The STZ-treated rats exhibited characteristic features of type I diabetes in that they failed to gain
weight, they suffered from hyperglycemia, and direct determination of fasting plasma insulin levels showed a substantial insulin deficiency. Moreover, the 8-wk diabetic state was associated with sensory neuropathy, which was reversible by insulin supplementation.

Diabetic neuropathy is a demonstrable disorder, either clinically evident or subclinical, that occurs in the setting of diabetes mellitus without other cause of peripheral neuropathy (25). As an experimental approach, STZ-induced diabetes has been extensively used to study the pathogenesis and consequences of diabetic neuropathy (5). In this model, neuropathy, similar to that seen in the type I diabetes, typically involves detrimental changes in sensory, autonomic, and motor nerves (12, 27). As far as the mechanism of diabetic sensory neuropathy is concerned, a defective axonal transport of sensory neuropeptides in addition to their decreased synthesis (24) is believed to be a critical initiating factor in degenerative distal neuropathies leading to severe microcirculatory changes (4, 9, 23). In addition, this possibly leads to a widespread deficiency in sensory effector function such as vasodilation, bronchomotility, or nonadrenergic, noncholinergic (NANC) contraction/relaxation (5, 15, 16, 17, 30, 33). In our main set of experiments, FS was used to study the effect of diabetes on nerve-mediated bronchoconstriction/relaxation. The results revealed that at least under our experimental conditions, the overall response to FS encompassed an initial atropine-sensitive contraction succeeded by a secondary longer-lasting contractile component resistant to atropine. These contractions were followed by relaxation, which also was atropine resistant. The latter two components, however, were blocked by prior systemic sequential capsaicin treatment, suggesting that they were of sensory neural origin. This treatment schedule has been shown to functionally deteriorate the majority of C fibers in rat (10). Experimental diabetes almost abolished the slow, atropine-insensitive contractile component and the relaxation component with less influence on the cholinergic response. Taking these results together with the decreased neuropeptide release from the bronchial preparations known to be densely innervated by CGRP and substance P-containing unmyelinated afferents that originate from the vagus nerve with cell bodies in the jugular, nodose, and dorsal root ganglia (28), we are not surprised that sensory neural dysfunction produced by diabetes was of significant influence on bronchomotor responses. Therefore, beyond the known impairment of cholinergic effector mechanisms in diabetes (3, 21), our present results strongly support the concept that a significant part of neural contractions of the bronchi is mediated by sen-
sory neuropeptides, the release/effect of which is attenuated by diabetes. This is in accord with findings by Gamse and Jancso (11) and that by Gyorfi et al. (12), that neurogenic inflammation, another process underlain by the local effector function of sensory nerves, is attenuated by STZ-diabetes. However, when either substance P or CGRP was added to preparations from diabetic rats, its potentiating effects on FS-induced contractions were not impaired. Moreover, FS-induced contractions in the presence of either peptide were significantly elevated compared with those seen in muscle rings from healthy animals. This means that neither the effect of these peptides nor the contractile responsiveness of bronchial smooth muscle was impaired by diabetes. In addition, it is also confirmed that these neuropeptides play a significant regulatory role in neural contractions of the bronchi. However, the inhibitory effect of exogenous somatostatin on FS-induced contractions was diminished in preparations from diabetic vs. normal animals. Therefore, it is speculated that there was a sensitization to CGRP and substance P effects and a desensitization to the effect of somatostatin in rings from diabetic rats. Interestingly, the plasma level of somatostatin was found to be increased in diabetic animals, similar to that found previously in 4-wk STZ-diabetes in rats (20), which may explain the dissociated tissue sensitivity to the neuropeptides studied.

To the best of our knowledge, this work is the first to show that the attenuated bronchomotor response in insulin-deficient diabetes is related to a decrease in sensory neuropeptide release. Because NANC contractile agents such as substance P and tachykinins play an important role in neurogenic bronchoconstriction (18), this means that bronchi of diabetic animals are less prone to contract in response to neural and antigen challenges. Beyond providing some approach as to why bronchial hyperreactivity is attenuated in diabetes, the results also call attention to pharmacological exploitation of the sensory neuropeptide release/effect- bronchial smooth muscle contraction pathway to confer protection on patients at risk of bronchial hyperreactivity.

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