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The effect of experimental diabetes on cholinergic neurotransmission in rat trachea: role of nitric oxide

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Abstract

We investigated the effect of nitric oxide (NO) on the responses of isolated tracheas to acetylcholine and to electrical field stimulation in streptozotocin-diabetic and controls rats. The contractile responses to acetylcholine were neither different nor affected by the NO synthase blocker, N^{ω}-nitro-L-arginine methyl ester (L-NAME), in the two groups. Diabetic rat tracheas were supersensitive to field stimulation. L-NAME enhanced field stimulation-induced contractions at low frequencies in control rat tracheas, but had no effect in diabetic rat tracheas. After L-NAME treatment, there was no difference in sensitivity to field stimulation between the groups. The relaxation responses to sodium nitroprusside in acetylcholine-precontracted tracheas were not different between the groups. However, diabetic rat trachea was supersensitive to the relaxant effect of sodium nitroprusside on contractile responses to field stimulation. These results suggested that the increase in sensitivity to field stimulation in tracheas from diabetic rats might be due to impairment in the production and/or release of an endogenous NO-like factor.

Keywords: Experimental diabetes; Cholinergic neurotransmission; Nitric oxide (NO)

1. Introduction

Streptozotocin-treated rats display symptoms similar to the clinical features of diabetes mellitus. Streptozotocin-diabetic rats have been widely used to study changes in the parasympathetic control of the gastrointestinal, cardiovascular and genitourinary systems during this disease process (Agrawal, 1980). The parasympathetic nervous system plays a dominant role in the control and regulation of the respiratory system in humans and in experimental animals. Cholinergic nerves provide the major bronchoconstrictor neural mechanisms in all species (Mann, 1971). However, the effects of diabetes on cholinergic motor transmission to the airways smooth muscle have not been adequately defined yet. The only study in the literature about the effect of diabetes on the cholinergic motor transmission to the isolated rat trachea, that by Cros et al. (1992), concluded that response to field stimulation was not specifically altered in diabetic rats.

Nitric oxide (NO) plays a role in the non-adrenergic non-cholinergic relaxation of tracheal smooth muscle in guinea-pigs (Tucker et al., 1990). Inhibitors of NO synthase (NOS) enhance cholinergic responses to field stimulation in guinea-pig trachea (Brave et al., 1991; Belvisi et al., 1993). Recently, it was also shown that an endogenous NO-like factor might mediate the inhibition of cholinergic contraction through a cyclic GMP-dependent mechanism in rat trachea (Sekizawa et al., 1993). There is evidence that streptozotocin-induced diabetes impairs nitrergic transmission in various tissues of rats, due to either a reduction in smooth muscle responsiveness to NO (Way and Reid, 1994) or to impairment in synthesis and/or release of NO (Wang et al., 1993; Jenkinson and Reid, 1995; Martinez-Cuesta et al., 1995; Kaputlu et al., 1999). It is also possible that the modulatory effect of NO on cholinergic contractions of rat trachea is impaired in diabetes and associated with possible changes in cholinergic neurotransmission to tracheal smooth muscle.

We have therefore investigated the responses of isolated tracheas to acetylcholine and to nerve stimulation and the modulatory effect of NO on these responses in streptozotocin-diabetic and non-diabetic controls rats.

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2. Methods

2.1. Treatment of animals

Male Wistar albino rats weighing 200–250 g were randomly allocated to two experimental groups each consisting of 12 animals. The animals were weighed and their weight was recorded. The rats to be made diabetic were lightly anaesthetized with diethyl ether and diabetes was then induced by a single intravenous injection of streptozotocin, 45 mg kg^{-1} via the tail vein. Streptozotocin was dissolved in 0.1 M citrate buffer (pH 4.5). The streptozotocin-treated rats received 2% sucrose in their drinking water for the first 48 h after treatment to reduce the severity of the initial hypoglycaemic phase following streptozotocin injection. Thereafter, streptozotocin-treated rats were maintained on standard rat chow and normal water ad libitum. Weight-matched control rats were injected with citrate buffer only and fed rat chow and plain water throughout the study. One week after streptozotocin, the blood glucose level was measured in a drop of blood obtained from a tail vein by puncturing the vein with a sterile needle using test strips (Glucostix, Bayer Diagnostics, Turkey) and glucometer (Glucometer® G_X, Bayer Diagnostics, Turkey). This was to ensure that diabetes had been induced. Animals with blood glucose levels ≥ 300 mg dl^{-1} were deemed to be diabetic and therefore suitable for the study.

2.2. Experimental procedures

Eight weeks after streptozotocin, after body weight and blood glucose level measurements, the rats were stunned and killed by decapitation. The trachea was removed rapidly and transverse rings (3 mm long) were cut and then mounted in thermostatically controlled (37°C) organ baths. The organ baths contained 20 ml Krebs–Henseleit solution (KHS) of the following composition (in mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $NaHCO_3$ 25.5, NaH_2PO_4 1.2, $CaCl_2$ 2.5 and glucose 5.6. The pH of the solution was 7.4 during bubbling with 5% CO_2 in O_2. Isometric tension was continuously measured with a force transducer (FDT10-A, Commat, Turkey), connected to a computer-based data acquisition system (TDA 97, Commat, Turkey). The tissues were stretched initially to a tension of 1 g for 30 s and thereafter maintained for 60 min under a resting tension of 0.5 g, which was found to be optimal for measuring the changes in tension. The preparations were washed with KHS every 15 min during the equilibration period. All experiments were carried out in the presence of indomethacin (1 μM), propranolol (1 μM) and phentolamine (10 μM). Indomethacin was used to preclude the generation of endogenous prostanoids that might alter responses to electrical field stimulation (Fernandes et al., 1994). The contractile responses induced under these conditions were completely abolished by tetrodotoxin (1 μM) or by atropine (1 μM), confirming the neural and cholinergic nature of the responses (data not shown).

2.3. Experiments on isolated tracheal rings

In each experiment, isolated tracheal rings were used to obtain contractile responses to two types of stimuli: electrical field stimulation and acetylcholine. The two types of stimuli were not delivered to the same ring. One, or if necessary, more rings were reserved for each type of stimulus. The parameters of field stimulation were as follows: supramaximal voltage of 80 V, 0.5 ms duration, 10-s train; 0.5, 1, 3, 5, 10, 20, 30 and 50 Hz at 2-min intervals. Electrical field stimulation was delivered by a Harvard Research Stimulator. Stimuli were delivered via two platinum electrodes (0.280 mm in diameter and 14 mm apart from each other) parallel to the tissue. Dose–response curves to acetylcholine (10^{-7}–10^{-3} M) were made cumulatively. Exposure to a dose of acetylcholine was maintained until the maximal response to that concentration of acetylcholine was obtained and then the tissue was exposed to the next, higher, concentration of acetylcholine.

In each tracheal ring, the concentration (EC_{50}) of acetylcholine and the frequency (EF_{50}) of field stimulation that produced approximately 50% of the maximal response were estimated. The effects of sodium nitroprusside (10^{-8}–10^{-4} M) on the contractile responses to EF_{50} of field stimulation (10–14 Hz for control and 5–9 Hz for diabetic group) in the absence and the presence of the NOS blocker, N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M), and to EC_{50} of acetylcholine (10^{-5} M for both groups), were studied. Sodium nitroprusside was added cumulatively to the bathing medium when the contractile response to acetylcholine reached a plateau. With field stimulation, each concentration of sodium nitroprusside was added to the bath after at least four stable responses (2-min interval) of equal magnitude had been obtained. Contractile responses to field stimulation were evoked until the maximal effect of the drug was observed.

In another set of experiments, we tested the effect of L-NAME (10^{-4} M) on the contractile responses to field stimulation and acetylcholine. L-NAME was added 20 min before the response to field stimulation or acetylcholine was elicited. In some experiments ($n = 8$), L-arginine (L-Arg, 1 mM,) was added 20 min before L-NAME to determine whether the effect of L-NAME was reversible and the inactive enantiomer, D-arginine (D-Arg, 1 mM), was used as a control.

2.4. Drugs

The following drugs were obtained from Sigma (St. Louis, MO, USA): streptozotocin, propranolol, phentolamine, tetrodotoxin, atropine sulphate, indomethacin, acetylcholine chloride, sodium nitroprusside, L-NAME, L-Arg and D-Arg. Solutions of drugs were made fresh on the

Table 1
Mean blood glucose (mg dl^{-1}) and body weight (g) of rats
Numbers in parentheses are the numbers of animals studied.

Group	Blood glucose	Body weight
Control (12)	115 ± 3	306 ± 18
Diabetic (12)	398 ± 8[a]	195 ± 12[a]

[a] $P < 0.05$: denotes significant difference from corresponding control.

day of their use with the exception of the tetrodotoxin solution which was stored at −20°C.

2.4.1. Preparation of drug solutions

Streptozotocin: this was dissolved immediately prior to its injection in 0.1 M citrate buffer (pH 4.5). The drug was kept on ice at all times before its use.

Tetrodotoxin: the stock solution of tetrodotoxin (10^{-3} M) was prepared in sodium citrate (pH 4.8). The working solutions were prepared fresh on the day of experiment by diluting the stock solution in distilled water.

Indomethacin: absolute ethanol was used to dissolve this drug to make a solution of 10^{-2} M.

All other drugs were dissolved in distilled water.

2.5. Analysis of results

At the end of each experiment, tracheal rings were detached from the recording setup, blotted and weighed. The contractile response was expressed as milligrams of tension developed per milligram of tissue wet weight. The relaxant response and decrease in amplitude of contractile responses were expressed as percentage of initial contraction. All values are expressed as means ± S.E.M. The logarithm of the concentration of agonist or frequency of field stimulation that elicited a 50% maximal response was designated as the EC_{50} or EF_{50}, respectively. These values were determined by regression analysis of the linear portions of the log concentration–response or of the log frequency–response curves. Sensitivity was expressed as pD_2 ($-\log EC_{50}$ or $\log EF_{50}$). Smooth muscle contractility was evaluated as the maximally developed tension per unit tissue weight (E_{max}). Statistical analysis of the results was performed using the analysis of variance and Student's *t*-test. *P* values lower than 0.05 were considered significant.

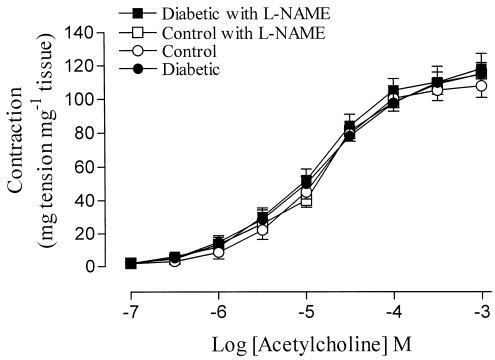

Fig. 1. Log concentration–response curves for acetylcholine in tracheal rings from control and diabetic rats in the absence and presence of L-NAME (10^{-4} M). L-NAME was added 20 min before the response to acetylcholine was elicited. $n = 12$ for all groups. Each point represents the mean with S.E.M. shown by vertical bars.

3. Results

Streptozotocin-diabetic rats had an elevated blood glucose level and a decreased body weight when compared with those of age-matched control rats (Table 1). Diabetic animals exhibited many other symptoms commonly associated with diabetes (e.g., polyuria, polydipsia and diarrhea).

There was no difference in sensitivity (pD_2) or maximal contractility (E_{max}) in response to acetylcholine between control and diabetic rat tracheas (Table 2, Fig. 1). In contrast, diabetic rat trachea demonstrated an increased sensitivity (pD_2) to electrical field stimulation. The maximum contractile response to field stimulation remained unchanged (Table 2, Fig. 2).

Administration of sodium nitroprusside caused a concentration-dependent relaxation of acetylcholine-precontracted tracheal rings and decreased the amplitude of the contractile responses to field stimulation in both control and diabetic rats. There was no difference in sensitivity or maximal relaxation in response to sodium nitroprusside between control and diabetic rat tracheas that were precon-

Table 2
pD_2 ($-\log EC_{50}$) and E_{max} (mg tension mg^{-1} tissue) values for acetylcholine and electrical field stimulation: effect of L-NAME
Numbers in parentheses are the numbers of animals studied.
L-NAME (10^{-4} M) was added 20 min before the response to acetylcholine or field stimulation was elicited.

Group	Without L-NAME-pretreatment				With L-NAME-pretreatment			
	Acetylcholine		Field stimulation		Acetylcholine		Field stimulation	
	pD_2	E_{max}	pD_2	E_{max}	pD_2	E_{max}	pD_2	E_{max}
Control (12)	4.87 ± 0.03	107.8 ± 7	1.086 ± 0.18	40.2 ± 4	4.78 ± 0.06	115 ± 7	0.833 ± 0.26[a]	42.6 ± 3
Diabetic (12)	4.85 ± 0.05	114.6 ± 9	0.839 ± 0.28[b]	40.3 ± 4	4.91 ± 0.04	118 ± 9	0.949 ± 0.19	44.2 ± 4

[a] $P < 0.05$: denotes significant difference from respective control value without L-NAME-pretreatment.
[b] $P < 0.05$: denotes significant difference from corresponding control.

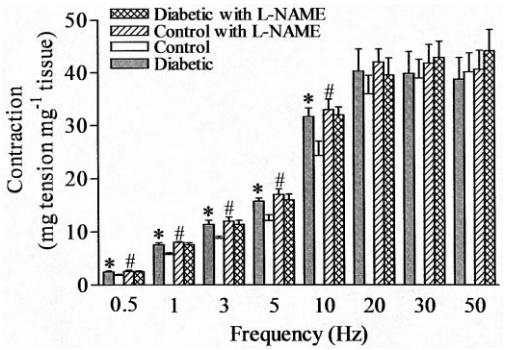

Fig. 2. Contractile responses to electrical field stimulation in tracheal rings from control and diabetic rats in the absence and presence of L-NAME (10^{-4} M). L-NAME was added 20 min before the response to field stimulation was elicited. $n = 12$ for all groups. Each point represents the mean with S.E.M. shown by vertical bars. $^{*}P < 0.05$ when compared to control group. $^{\#}P < 0.05$ when compared to respective control value without L-NAME-pretreatment.

tracted with EC_{50} of acetylcholine. In contrast, tracheas from streptozotocin-diabetic rats were supersensitive to the inhibitory effect of sodium nitroprusside on contractile responses produced by EF_{50} of field stimulation; the pD_2 value for sodium nitroprusside was significantly higher in tracheal rings from streptozotocin-diabetic rats than in tracheal rings from control animals. However, the maximal effect of sodium nitroprusside remained unchanged. L-NAME (10^{-4} M) pretreatment caused a significant increase in sensitivity to the inhibitory effect of sodium nitroprusside on contractile responses to field stimulation in tracheas from control rats, whereas it did not cause a further increase in sensitivity to sodium nitroprusside in diabetic rat tracheas. The maximal effects of sodium nitroprusside remained unchanged in both groups. (Table 3, Fig. 3).

Exposure of tracheal rings to L-NAME (10^{-4} M) caused slight contractions in both control and diabetic rat tracheas. The contractions elicited by L-NAME tended to be smaller in diabetic than in control rat trachea (7.2 ± 3 vs. 12.7 ± 4 mg tension mg^{-1} tissue, respectively). L-Arg and D-Arg (1 mM) had no effect on resting tone. L-NAME pretreatment significantly enhanced field stimulation-induced contractions at low frequencies (0.5–10 Hz) in control rat tracheas

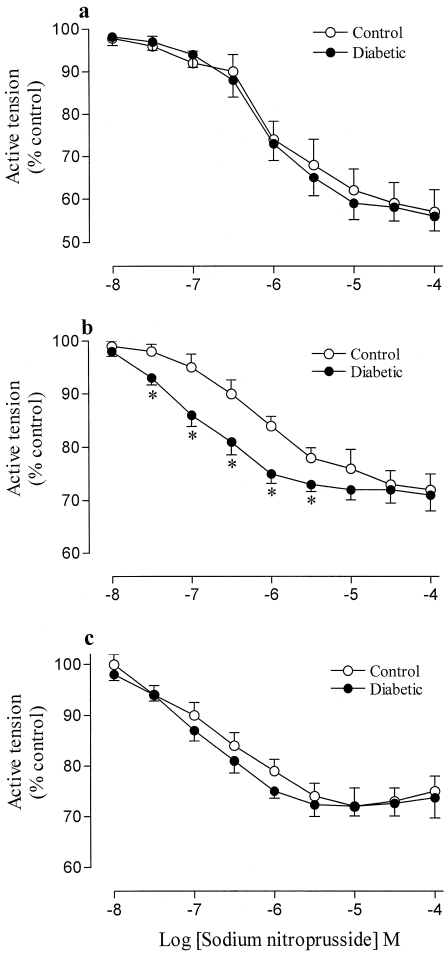

Fig. 3. Log concentration–response curves to sodium nitroprusside in the contractile responses to EC_{50} of acetylcholine (a) and EF_{50} of electrical field stimulation (b,c), in the absence (b) and the presence (c) of L-NAME (10^{-4} M) in tracheal rings from control and diabetic rats. $n = 12$ (a,b) and 6 (c) for both groups. Each point represents the mean with S.E.M. shown by vertical bars. $^{*}P < 0.05$ when compared to their respective controls.

(Fig. 2). This effect of L-NAME was partially reversed by L-Arg (log EF_{50} for field stimulation increased from 0.833 $\pm$ 0.26 to 0.973 ± 0.08, $P < 0.05$, $n = 8$), but not by D-Arg. Although L-NAME caused slight increases in field stimulation-induced contractile responses in diabetic rat

Table 3
Sodium nitroprusside pD_2 ($-\log EC_{50}$) and E_{max} (active tension, percentage control) values for contractions in response to EC_{50} of acetylcholine and EF_{50} of electrical field stimulation.
Numbers in parentheses are the numbers of animals studied for both groups.
L-NAME (10^{-4} M) was added 20 min before the response to sodium nitroprusside was elicited.

Group	On acetylcholine (EC_{50}) (12)		On field stimulation (EF_{50})			
	pD_2	E_{max}	Without L-NAME (12)		With L-NAME (6)	
			pD_2	E_{max}	pD_2	E_{max}
Control	6.05 ± 0.04	57.2 ± 3	6.15 ± 0.06	72.4 ± 3	6.71 ± 0.11^{a}	72.3 ± 2
Diabetic	6.12 ± 0.08	56.4 ± 3	6.91 ± 0.07^{b}	71.0 ± 3	6.92 ± 0.07	73.3 ± 4

[a] $P < 0.05$: denotes significant difference from respective control value without L-NAME-pretreatment.
[b] $P < 0.05$: denotes significant difference from corresponding control.

tracheas, they were not statistically significant and not affected significantly by either L- or D-Arg. After L-NAME treatment, there was no difference in sensitivity and maximal contractile response to field stimulation between tracheal rings of control and diabetic rats (Table 2, Fig. 2). In contrast to field stimulation-induced contractions, acetylcholine-induced contractions were not significantly affected by L-NAME-pretreatment (Table 2, Fig. 1).

4. Discussion

There are sound reasons to believe that the animals treated with streptozotocin in the present study were fully diabetic. Data shown in Table 1 demonstrating the failure of the streptozotocin-treated rats to gain weight together with the highly significant rise in blood glucose levels and the increase in urine output in these animals are strongly indicative of the successful induction of diabetes.

The present results demonstrated that tracheal sensitivity (log EF_{50}) to electrical field stimulation was enhanced, but that the maximal response (E_{max}) did not change in 8-week diabetic rats compared to that in control rats. There is a lack of basic research on the effect of diabetes on nerve-mediated cholinergic responses of trachea. The only study concerning this issue, that by Cros et al. (1992), concluded, in contrast to what we found, that the response to field stimulation was not specifically altered in trachea from diabetic rats.

Conflicting results have been reported in the literature with regard to the sensitivity (Cros et al., 1992; Ozansoy et al., 1993a,b) and the maximal contractile responses (Mongold et al, 1988; Cros et al., 1992; Ozansoy et al., 1993a,b) to acetylcholine receptor agonists in tracheas from rats that were diabetic for different durations. The most likely explanation for the differences in sensitivity and maximal responses to acetylcholine receptor agonists seems to be the state of innervation of the trachea, which in turn would depend upon the severity and duration of experimental diabetes. This view was further supported by Cros et al. (1992) who proposed that there is a major time-dependence in tracheal responsiveness during diabetes.

Latifpour et al. (1989) suggested that there is a clear correlation between muscarinic receptor upregulation and contractile supersensitivity. Although we did not investigate the effect of diabetes on the biochemical characteristics of muscarinic receptors in rat trachea, the binding studies of lung cholinergic receptors, using the antagonist ligand, [^{3}H]quinuclidinyl benzylate, and the agonist, carbachol, did not detect any change in diabetic compared to control rats (Cros et al., 1992), which further supported our finding of unchanged sensitivity and maximal response to acetylcholine in tracheas from diabetic rats. Contrastingly, Belmonte et al. (1997) showed that the neuronal M_2 muscarinic receptors in the lungs that inhibit acetylcholine release had an increased function in diabetic rats, associated with an increase in agonist affinity.

In theory, the increase in sensitivity of diabetic rat trachea to a nerve-mediated cholinergic response must have originated in either augmented sensitivity of the trachea to released acetylcholine or to an augmented acetylcholine release or to a combination of both these factors. In the present study, similar sensitivity and maximal responses of diabetic and control rat tracheas to exogenously applied acetylcholine excluded the possibility of a postsynaptic exaggerated responsiveness or supersensitivity to released acetylcholine. The quantum of neurotransmitter released in response to the stimulus and the rate of acetylcholine inactivation are the main factors determining the transmitter concentration in the synaptic cleft (Luneshi and Zar, 1991). Although we did not measure tissue cholinesterase activity, the possibility that the transmitter concentration within the synaptic cleft might be higher in diabetic rat trachea due to a diminished rate of acetylcholine inactivation seems unlikely, since the contractile responses of tracheas to exogenously applied acetylcholine did not differ significantly between diabetic and control rats at any concentration of acetylcholine. It follows, therefore, that the likeliest cause of the increase in sensitivity to nerve-mediated cholinergic responses in the diabetic trachea is a greater than normal release of acetylcholine in response to field stimulation at low frequencies.

Another possible mechanism for the enhanced sensitivity to field stimulation in diabetic rat trachea might be an increase in the release of vasoconstrictors or a reduction in the release of and/or reactivity of tracheal smooth muscle to vasodilators. Endogenous prostanoids would be possible candidates (Fernandes et al., 1994). However, this possibility was excluded in the present study by the presence of indomethacin (1 μM) in KHS throughout the experiment. On the other hand, Sekizawa et al. (1993) suggested that an endogenous NO-like factor released during field stimulation might mediate prejunctional inhibition of cholinergic contraction through a cyclic GMP-dependent mechanism in rat trachea. Similarly, inhibitors of NOS enhanced the cholinergic responses to field stimulation in guinea-pig trachea (Brave et al., 1991; Belvisi et al., 1993). Therefore, it is possible that the increase in sensitivity to field stimulation in tracheas from diabetic rats might be due to a reduced production and/or release of this endogenous NO-like factor or to a reduction in the response of tracheal smooth muscle from diabetic rats to the relaxing effect of this substance. In the present study, L-NAME pretreatment abolished the difference in sensitivity to field stimulation between diabetic and control rat tracheas mainly by enhancing the field stimulation-induced contractions in control rats (Fig. 2). The effect of L-NAME was partially reversed by L-Arg, but not D-Arg, which is not a substrate for NOS. Neither L-NAME nor L-Arg exerted any significant effect on responses to field stimulation in diabetic rats. Furthermore, the contractions induced by exposure of

tracheal rings to L-NAME tended to be smaller in diabetic than in control rats. Taken together, these findings indicated that the reductions in production and/or release of an endogenous NO-like factor might be responsible for the increased tracheal sensitivity to field stimulation in diabetic rats. In agreement with this view, impairment in synthesis and/or release of NO was also reported for various tissues of diabetic rats (Chang and Stevens, 1992; Wang et al., 1993; Jenkinson and Reid, 1995; Martinez-Cuesta et al., 1995; Kaputlu et al., 1999).

In the present study, diabetic rat tracheas were more sensitive to the relaxant effect of sodium nitroprusside, an agent-releasing NO (Moncada, 1992), on field stimulation-induced contractions and the maximal relaxations in response to sodium nitroprusside were not significantly different between diabetic and control rat tracheas. Moreover, there was no significant difference in relaxant effect of sodium nitroprusside on acetylcholine-precontracted tracheas from diabetic and control rats, which further excluded the possibility of impairment of the tracheal smooth muscle response to NO in diabetic rats. Furthermore, a significant leftward shift of the sodium nitroprusside dose–response curve indicating possible supersensitivity of cholinergic nerve terminals to NO in tracheas from diabetic rats was also observed in tracheas from control rats in the presence of L-NAME. L-NAME pretreatment did not cause a further increase in sensitivity to sodium nitroprusside in tracheas from control rats. In the presence of NOS inhibitors, enhanced responses to sodium nitroprusside and other nitroso compounds related to an increased sensitivity to exogenous NO when endogenous NO production was reduced, have also been shown in vascular tissue (Gardiner et al., 1991; Moncada et al., 1991). Therefore, it might be speculated that a reduction in endogenous NO production and/or release in diabetes may also be responsible for the observed increase in sensitivity of cholinergic nerve terminals to sodium nitroprusside in rat trachea.

The source of NO released during field stimulation in the present study is unknown. As L-NAME pretreatment enhanced cholinergic responses to field stimulation without affecting the contractile responses to exogenous acetylcholine (Fig. 1) and sodium nitroprusside caused a relaxant effect on tracheas precontracted with acetylcholine, it might be suggested that NO production and/or release must be caused by stimulation of nitrergic nerves. The site of action of NO to inhibit acetylcholine release may be prejunctional, thereby reducing cholinergic response. However, it was also suggested that NO modulates cholinergic neurotransmission via relaxation of airway smooth muscle, which functionally antagonises the effect of acetylcholine released from cholinergic nerves (Brave et al., 1991). Therefore, it might be suggested that a reduction in production and/or release of NO in tracheas from diabetic rats may result in both augmented acetylcholine release following field stimulation and impairment in functional antagonism of released acetylcholine. This could explain both the increased sensitivity and unchanged maximal response to field stimulation in tracheas from diabetic rats, given that NO is preferentially released at low stimulation frequencies (Li and Rand, 1990; D'Amato et al., 1992).

In summary and conclusion, the present study has shown that the sensitivity to electrical field stimulation was enhanced in tracheas from 8-week diabetic rats, but that the responses to exogenous acetylcholine did not change. It is suggested that the increase in sensitivity to field stimulation in tracheas from diabetic rats might be due to impairment in production and/or release of an endogenous NO-like factor during cholinergic nerve stimulation.

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