Role of insulin in antigen-induced airway eosinophilia and neuronal M$_2$ muscarinic receptor dysfunction

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Belmonte, K. E., A. D. Fryer, and R. W. Costello. Role of insulin in antigen-induced airway eosinophilia and neuronal M$_2$ muscarinic receptor dysfunction. J. Appl. Physiol. 85(5): 1708–1718, 1998.—In the lungs, neuronal M$_2$ muscarinic receptors limit ACh release from parasympathetic nerves. In antigen-challenged animals, eosinophil proteins block these receptors, resulting in increased ACh release and vagally mediated hyperresponsiveness. In contrast, diabetic rats are hyporesponsive and have increased M$_2$ receptor function. Because there is a low incidence of asthma among diabetic patients, we investigated whether diabetes protects neuronal M$_2$ receptor function in antigen-challenged rats. Antigen challenge of sensitized rats decreased M$_2$ receptor function, increased vagally mediated hyperresponsiveness by 75%, and caused a 10-fold increase in eosinophil accumulation around airway nerves. In antigen-challenged diabetic rats, neuronal M$_2$ receptor function was preserved and there was no eosinophil accumulation around airway nerves. Insulin treatment of diabetic rats completely restored loss of M$_2$ receptor function, vagally mediated hyperresponsiveness, and eosinophilia after antigen challenge. These data demonstrate that insulin is required for development of airway inflammation, loss of neuronal M$_2$ muscarinic receptor function, and subsequent hyperresponsiveness in antigen-challenged rats and may explain decreased incidence of asthma among diabetic humans.

airway hyperreactivity; airway inflammation; diabetes; asthma; vagus nerves

In the lungs, vagal parasympathetic nerves release ACh, which stimulates M$_3$ muscarinic receptors on airway smooth muscle, causing bronchoconstriction (31). At the same time, ACh stimulates inhibitory M$_2$ muscarinic receptors on the parasympathetic nerves, limiting further ACh release (16). The importance of these neuronal M$_2$ muscarinic autoreceptors in control of ACh release can be demonstrated with muscarinic antagonists and agonists. Blockade of neuronal M$_2$ muscarinic receptors with the selective muscarinic antagonist gallamine potentiates vagally induced bronchoconstriction as much as five- to eightfold, whereas stimulation of neuronal M$_2$ muscarinic receptors with the muscarinic agonist pilocarpine inhibits vagally induced bronchoconstriction by as much as 70% (16). Thus neuronal M$_2$ muscarinic receptors exert an important local control over ACh release and over vagally induced bronchoconstriction.

Neuronal M$_2$ muscarinic receptors are dysfunctional in humans with asthma (5, 28) and in antigen-challenged guinea pigs (17), resulting in increased bronchoconstriction in response to vagal stimulation. Because the parasympathetic nerves provide the dominant control of airway smooth muscle tone, loss of neuronal inhibitory M$_2$ receptor function may be important in the development of airway hyperresponsiveness in antigen-challenged animals and in humans with asthma (10).

A low incidence of asthma among patients with diabetes mellitus has been reported in a number of epidemiologic studies (2, 22, 34), although the mechanism underlying this protection is unclear. In contrast to the airway hyperreactivity characteristic of asthma, the airways of patients with diabetes mellitus are characterized by increased neuronal inhibitory M$_2$ muscarinic receptors (6). Insulin is required for development of airway inflammation, loss of neuronal M$_2$ muscarinic receptor function, and subsequent hyperresponsiveness in antigen-challenged guinea pigs. In contrast, diabetes is associated with decreased allergic inflammation, particularly of eosinophils (3, 18, 35).

We have shown that hyperreactivity is mediated by the recruitment of eosinophils to the airway nerves and subsequent loss of neuronal M$_2$ muscarinic receptor function. Diabetes is associated with decreased reactivity. These studies were carried out to determine whether decreased insulin, as seen in diabetes, is a mechanism for inhibition of recruitment of eosinophils to the nerves. This would result in protection of neuronal M$_2$ muscarinic receptor function and inhibition of hyperreactivity in antigen-challenged rats.

METHODS

Animals. Male Sprague-Dawley pathogen-free rats (250-300 g; Hilltop Animal Farms, Scottsdale, PA) were shipped in...
filtered crates and housed in wire-bottomed cages inside laminar flow hoods. Masks and gloves were worn by personnel entering the room or handling the rats. All rats were handled in accordance with the standards established by the US Animal Welfare Act set forth in the National Institutes of Health guidelines and the Policy and Procedures Manual published by the Johns Hopkins University School of Hygiene and Public Health Animal Care and Use Committee.

Rats were divided into the following treatment groups: 1) diabetic, 2) antigen sensitized and challenged, 3) diabetic, antigen sensitized, and challenged, 4) insulin-treated, diabetic, antigen sensitized, and challenged, and 5) unchallenged and nondiabetic (controls).

Induction of diabetes. Diabetes mellitus was induced by a single injection of streptozotocin (STZ, 65 mg/kg dissolved in 0.1 M sodium citrate buffer, pH 4.5) into the tail vein. The presence of glycosuria was tested 24 h postinjection, and diabetes was confirmed before each experiment by the presence of severe hyperglycemia in whole blood (>275 mg/dl). Control rats were injected with the same volume (0.3 ml) of citrate buffer into the tail vein.

Glucose measurement. Glucose was measured in urine by Tes-tape (Eli Lilly, Indianapolis, IN) and in whole blood by a standard glucometer (Encore, Miles, Elkhart, IL) before each experiment.

Antigen sensitization and challenge. Rats were sensitized on days 1, 3, and 7 using an adaptation of a previously reported sensitization procedure (20). A solution of ovalbumin (10 mg/kg) and aluminum hydroxide (100 mg/kg) in 1 ml of PBS was given subcutaneously. At the same time 1 ml/kg of the same ovalbumin + aluminum hydroxide solution mixed with 0.5 ml of heat-killed Bordetella pertussis vaccine (1 × 10^9 cells/ml) suspended in PBS was given intraperitoneally. Some of the antigen-sensitized rats were made diabetic by STZ (see above) 7–14 days before antigen challenge. Twenty-one days after sensitization, animals were challenged in a laminar flow hood with an aerosolized solution of 5% ovalbumin for 5 min. In the STZ-treated rats, diabetes was confirmed immediately before antigen challenge by the presence of glycosuria. Experiments were performed 18–24 h after the aerosol challenge.

Insulin administration. Some of the antigen-sensitized diabetic rats received a long-acting (NPH) insulin (2 U/day sc, I sophane, Eli Lilly) for 7 days, beginning 7 days after the STZ injection (day 14) and ending the day before antigen challenge (day 20).

Confirmation of antigen sensitization. Antigen sensitization was confirmed at the end of each experiment by administration of ovalbumin (250 mg/kg iv) in PBS to induce anaphylaxis.

Anesthesia, surgical preparation, and measurement of pulmonary inflation pressure. Rats were anesthetized with urethane (1.5 g/kg ip). This dose produces a deep, 8- to 10-h anesthesia (19), although no experiment lasted >3 h. Once the rats were anesthetized, the carotid artery was cannulated for the measurement of heart rate and blood pressure and a jugular vein was cannulated for the administration of intravenous drugs. The animals were paralyzed with suxamethonium (30 mg/kg iv, given at 30-min intervals). The trachea was cannulated, and the animals were ventilated with a positive-pressure, constant-volume respirator (Harvard Apparatus, S. Natick, MA; tidal volume 5 ml/kg, 100 breaths/min). Both vagi were isolated and cut, and the distal portions were placed on shielded platinum electrodes and immersed in a pool of liquid paraffin. The animal's body temperature was maintained at 37°C with a homeothermic blanket (Harvard Apparatus).

Pulmonary inflation pressure was measured at the trachea with a pressure transducer (model DTX, Spectromed), and all signals were recorded on a polygraph (Grass Instrument, Quincy, MA). A positive pressure of 95–120 mmH₂O was necessary to ventilate the animals adequately. This value was taken as the baseline pulmonary inflation pressure. Bronchoconstriction was measured as the increase in pulmonary inflation pressure above the baseline inflation pressure, as previously described (16).

All animals were treated with propranolol (1 mg/kg) before each experiment to block the effects of catecholamines. Simultaneous stimulation of both vagus nerves (30 Hz, 0.4-ms pulse duration, 180 pulses/train; SD9 stimulator, Grass Instrument), at 1-min intervals, caused bronchoconstriction and bradycardia. Both of these vagally mediated responses were rapidly reversed at the termination of each stimulus.

Testing function of neuronal M₂ muscarinic receptors. Vaguely induced bronchoconstriction was matched between the groups by increasing or decreasing the voltage of vagal stimulation over a range of 20–40 V. Once reproducible bronchoconstriction was achieved, the effect of pilocarpine (0.001–100 µg/kg iv) on vagally induced bronchoconstriction was measured and calculated as the percent change in vagally induced bronchoconstriction compared with the bronchoconstriction before pilocarpine. At the end of each experiment, vagally induced bronchoconstriction and bradycardia were abolished by atropine (1 mg/kg iv), confirming that vagally induced bronchoconstriction was mediated by ACh-stimulating muscarinic receptors.

Neutralization of cationic proteins with heparin. After the dose-response curve to pilocarpine, those antigen-challenged animals with a bronchoconstriction of ≥70% of the initial bronchoconstriction after receiving 100 µg/kg of pilocarpine were deemed to have "dysfunctional" neuronal inhibitory M₂ muscarinic receptors, and so they were given the polyanionic compound heparin (3,000 U iv). Heparin has been shown to neutralize cationic proteins in antigen-challenged guinea pigs in vivo, thereby acutely restoring function of neuronal M₂ muscarinic receptors (15). The ability of heparin to reduce vagally induced bronchoconstriction was measured 5–15 min after administration.

Testing function of M₃ muscarinic receptors on airway smooth muscle. In some experiments the vagus nerves were cut, and bronchoconstriction and bradycardia were induced by intravenous ACh (1–800 µg/kg). At the end of each experiment, all ACh-induced responses were abolished by atropine (1 mg/kg iv), confirming that vagally induced bronchoconstriction was mediated by muscarinic receptors.

Assessment of airway reactivity. To measure airway reactivity and to assess the contribution of the vagus nerves to airway reactivity, bronchoconstriction was induced by methacholine (1–20 µg/kg iv) in anesthetized rats with the vagi intact and then repeated with the vagi cut.

Measurement of inflammatory cells. After each experiment the lungs were lavaged with five separate 10-ml aliquots of PBS with isoproterenol (10⁻⁴ M). The recovered bronchoalveolar lavage fluid was centrifuged (400 g, 10 min), and the pellet was resuspended in PBS. Cells were counted on a hemocytometer. Differential cell counts were performed by centrifuging the cells onto slides (Cytospin 3, Shandon, Pittsburgh, PA) and then staining with Diff-Quik (American Scientific Products).

Histological preparation. In some animals the chest was opened at the termination of the in vivo studies, while the animals were still under deep anesthesia. The animal was killed by exsanguination, and the lungs were removed and inflated with 10 ml/kg of 4% paraformaldehyde in 0.1 M
phosphate buffer; this volume is equivalent to the rat’s functional residual capacity. A section from each lobe of lung was then embedded in paraffin.

Identification of airway nerves using protein gene product 9.5. The polyclonal rabbit antibody to protein gene product 9.5 (PGP 9.5) (37) was used to identify airway nerves. Sections of paraffin-embedded tissue, 6 µm thick, were dewaxed by immersion in xylene, rehydrated through a graded alcohol series, and washed three times in Tris-HCl (pH 7.8). Nonspecific peroxidase was inhibited by applying a solution of 3% hydrogen peroxide in methanol for 10 min, then washing three times for 5 min each with Tris-HCl. The tissue was then overlaid with 10% normal goat serum for 30 min, and rabbit anti-PGP 9.5 (1:4,000) was applied to the tissue for 36 h at 4°C. The tissue was washed three times in Tris-HCl over 15 min, and a biotinylated goat anti-rabbit antibody (Vector, Burlingame, CA) was added (1:50) for 30 min. The tissue was rewash ed three times over 15 min with Tris-HCl, and an avidin-horseradish peroxidase complex was added for 30 min (ABC Elite, Vector, Burlingame, CA). The antibody was detected by the addition of chromagen SG (Vector). In the presence of hydrogen peroxide, chromagen SG stained the nerves black. Rabbit serum absorbed over PGP 9.5 was used as a control antibody. The sections were stained in Luna’s solution, 1% chromotrope 2R to identify eosinophils, or a 0.01% aqueous solution of toluidine blue to identify mast cells (32). Additional sections were stained with hematoxylin and eosin to identify all inflammatory cells.

Histological analysis. Four airways from each of three animals representing each experimental group were examined in a blinded manner (12 airways/group). Airways were selected by starting at the top left corner of the slide and moving in a counterclockwise manner. The airways were photographed, and the images were transferred to a computer for image analysis (Image Pro Plus). The total number of eosinophils and mast cells per unit area (µm²) of each airway were compared between each experimental group. Similarly the number of eosinophils and mast cells within 8 µm of a nerve per airway were compared between groups.

Statistics. Values are means ± SE. The responses to intravenous ACh and methacholine and the effects of pilocarpine on vagally induced bronchoconstriction in the various experimental groups were compared using two-way ANOVA for repeated measures with Fisher’s correction. Baseline responses to vagal stimulation, baseline pulmonary inflation pressure, baseline heart rate, baseline blood glucose levels, and differences in inflammatory cells were compared between the groups using ANOVA. P < 0.05 was considered statistically significant.

Drugs and reagents. ACh, atropine, chromotrope 2R, heparin, methacholine, ovalbumin, pilocarpine, propranolol, suxamethonium, STZ, and urethran were purchased from Sigma Chemical (St. Louis, MO). Aluminum hydroxide was purchased from EM Science (Gibbstown, NJ). NPH Iletin II isophane insulin suspension was purchased from Eli Lilly. Rabbit anti-PGP 9.5 and rabbit serum absorbed over PGP 9.5 were purchased from Biogenex (Sunnyvale, CA). Heat-killed B. pertussis vaccine was purchased from the Michigan Department of Public Health (Lansing, MI). All drugs were dissolved in 0.9% NaCl or PBS, except STZ, which was dissolved in 0.1 M sodium citrate buffer, pH 4.5.

RESULTS

The baseline pulmonary inflation pressure, heat rate, and blood glucose values for each experimental group are outlined in Table 1. With the vagus nerves cut, baseline pulmonary inflation pressure was not significantly different between any of the groups. The baseline heart rate was significantly lower in diabetic and antigen-challenged diabetic rats than in controls (both groups P = 0.04 compared with controls). The blood glucose levels were significantly elevated in all diabetic rats compared with controls.

Some antigen-sensitized diabetic rats received insulin at 2 U/day for 7 days before antigen challenge. In insulin-treated diabetic antigen-challenged rats, no change was noted in baseline pulmonary inflation pressure or heart rate, but blood glucose levels remained significantly elevated compared with controls (Table 1). Thus, despite administration of exogenous insulin to diabetic rats, blood glucose levels in insulin-treated rats were not significantly different from blood glucose levels in diabetic rats.

Bronchoconstriction induced by intravenous methacholine. Methacholine (1–20 µg/kg iv) produced dose-related bronchoconstriction, measured as increases in pulmonary inflation pressure, in all groups of animals. When the vagus nerves were intact, the dose-response curves to methacholine were significantly higher than after the vagus nerves were cut in all experimental groups (cf. Fig. 1A with Fig. 2A and Fig. 1B with Fig. 2B). Preliminary studies showed no evidence of tachyphylaxis to methacholine in control or challenged rats (n = 3). Thus methacholine-induced bronchoconstriction is due to a direct effect on the muscle and an indirect, vagally mediated reflex. This is further supported by the observation that pilocarpine inhibits methacholine-induced bronchoconstriction (data not shown).

In antigen-challenged rats, methacholine-induced bronchoconstriction was significantly increased compared with controls when the vagus nerves were intact (Fig. 1A; P = 0.001). When the vagus nerves were cut, there were no differences in the bronchoconstriction response to methacholine between antigen-challenged and control rats (Fig. 1B).

In diabetic rats, methacholine-induced bronchoconstriction was slightly, but not significantly, depressed compared with controls when the vagus nerves were intact (Fig. 1A). When the vagus nerves were cut, methacholine-induced bronchoconstriction was reduced in diabetic and control rats, and the dose-

Table 1. Baseline pulmonary inflation pressure, heart rate, and blood glucose levels

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Ppi, mmH2O</th>
<th>Heart Rate, beats/min</th>
<th>Blood Glucose, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>127.0 ± 5.9</td>
<td>386.3 ± 11.4</td>
<td>162.1 ± 19.7</td>
</tr>
<tr>
<td>Diabetic</td>
<td>127.8 ± 5.6</td>
<td>343.3 ± 13.4*</td>
<td>476.8 ± 25.1*</td>
</tr>
<tr>
<td>Antigen-challenged</td>
<td>123.3 ± 4.2</td>
<td>375.0 ± 5.4</td>
<td>158.6 ± 22.0</td>
</tr>
<tr>
<td>Diabetic antigen-challenged</td>
<td>125.0 ± 8.9</td>
<td>336.7 ± 21.0*</td>
<td>481.1 ± 26.3*</td>
</tr>
<tr>
<td>Insulin-treated antigen-challenged</td>
<td>118.2 ± 4.5</td>
<td>348.2 ± 8.6</td>
<td>419.0 ± 41.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE of 6–10 animals in each group. Ppi, pulmonary inflation pressure. *Significantly different from control (P = 0.05).
response curves were not significantly different from each other (Fig. 1B).

In contrast to antigen-challenged rats, methacholine-induced bronchoconstriction in diabetic antigen-challenged rats with the vagi intact was the same as in controls with the vagi intact (Fig. 2A). When the vagi were intact, methacholine-induced bronchoconstriction in insulin-treated diabetic antigen-challenged rats was increased, so that it was significantly greater than in controls (Fig. 2A) and not significantly different from that in antigen-challenged rats (cf. Fig. 2A with Fig. 1A). After the vagi were cut, the dose response to methacholine was the same among control, diabetic antigen-challenged, and insulin-treated diabetic antigen-challenged rats (Fig. 2B).

Bronchoconstriction induced by intravenous ACh. ACh (1–800 µg/kg iv) produced dose-related bronchoconstriction, measured as increases in pulmonary inflation pressure in all groups (control, diabetic, antigen-challenged, diabetic antigen-challenged, and insulin-treated diabetic antigen-challenged rats) with the vagi cut. ACh-induced bronchoconstriction was not significantly different among any of the groups (Fig. 3).

Effect of pilocarpine on vagally induced bronchoconstriction. Simultaneous electrical stimulation of the distal ends of the cut vagus nerves (30 Hz, 0.4 ms, 180 pulses/train) caused bronchoconstriction and bradycardia. Because no difference was seen in the bronchoconstrictor response to exogenous ACh among all the groups tested, the magnitude of bronchoconstriction was assumed to be a function of the concentration of ACh at the neuromuscular junction. Thus vagally induced bronchoconstriction in the absence of pilocarpine was matched by adjusting the voltage over a range of 20–40 V so that it was not significantly different between any of the groups (26.7 ± 3.7, 22.9 ± 1.4, 24.1 ± 2.6, 24.3 ± 1.8, and 29.2 ± 1.5 mm H2O in control, antigen-challenged, diabetic, diabetic antigen-challenged, and insulin-treated diabetic antigen-challenged rats, respectively). The function of neuronal M2 muscarinic receptors was then tested using the muscarinic agonist pilocarpine (0.001–100 µg/kg iv).

In control rats, pilocarpine inhibited vagally induced bronchoconstriction in a dose-related manner (Fig. 4). In diabetic rats, pilocarpine inhibited vagally induced bronchoconstriction to a significantly greater degree...
than in control animals. The dose-response curve to pilocarpine was shifted to the left and downward compared with controls (Fig. 4; \( P < 0.008 \)). In contrast, pilocarpine did not inhibit vagally induced bronchoconstriction in antigen-challenged rats (Fig. 4; \( P < 0.001 \)).

Although pilocarpine did not inhibit vagally induced bronchoconstriction in antigen-challenged rats, pilocarpine did inhibit vagally induced bronchoconstriction in diabetic antigen-challenged rats (Fig. 5; \( P = 0.001 \)). The response to pilocarpine in the diabetic antigen-challenged group was not different from controls (Fig. 5). In insulin-treated diabetic antigen-challenged rats, pilocarpine did not inhibit vagally induced bronchoconstriction (Fig. 5; \( P = 0.001 \)). The response to pilocarpine in the insulin-treated diabetic antigen-challenged group was not different from that in the antigen-challenged rats (cf. Fig. 5 with Fig. 4).

Effect of heparin on vagally induced bronchoconstriction. Heparin (3,000 U iv) was given to those antigen-challenged animals in which pilocarpine at 100 µg/kg iv did not significantly inhibit vagally induced bronchoconstriction (Figs. 4 and 5). In the presence of pilocarpine, heparin inhibited vagally induced bronchoconstriction, reducing the response to 36% of the bronchoconstriction in the absence of pilocarpine (a fall of 64%). Administration of heparin to control, nonchallenged rats did not affect vagally induced bronchoconstriction (n = 2, data not shown).

Assessment of inflammation by bronchoalveolar lavage. Diabetes decreased the total number of cells recovered by bronchoalveolar lavage compared with controls, whether or not the animals were also antigen challenged (Fig. 6A). Antigen challenge alone did not alter the total number of inflammatory cells compared with controls. Insulin treatment of the antigen-challenged diabetic rats prevented the decrease in the total number of inflammatory cells seen with diabetes. There were few eosinophils recovered by bronchoalveolar lavage from the lungs of control animals (Fig. 6B).
This number of eosinophils was slightly, but not significantly, reduced by diabetes. In contrast, eosinophils were significantly increased in bronchoalveolar lavage from antigen-challenged rats compared with controls. Eosinophil numbers were not increased in diabetic antigen-challenged rats but were increased in insulin-treated diabetic antigen-challenged rats (Fig. 6B).

The effects of antigen challenge and diabetes on macrophages, neutrophils, and lymphocytes were similar to the effect on eosinophils. All three cell populations were decreased by diabetes and slightly increased by antigen challenge (except for macrophages, which did not change with antigen challenge). In diabetic antigen-challenged rats, numbers of macrophages, neutrophils, and lymphocytes were significantly reduced compared with antigen-challenged rats. In insulin-treated diabetic antigen-challenged rats, populations of macrophages, neutrophils, and lymphocytes were restored to values seen in antigen-challenged rats (Fig. 7).
Histological assessment of airway eosinophilia. Histological examination of rat airways showed that eosinophils were rarely seen in the bronchi and, in particular, were rarely associated with the airway nerves of control (Fig. 8, A and B) or diabetic rats (Fig. 8, C and D). In contrast, eosinophils were present in the airways and in association with the nerves in antigen-challenged rats (arrows, Fig. 8, E and F). There was no influx of eosinophils in diabetic antigen-challenged rats (Fig. 8, G and H), unless they were treated with insulin, which restored the influx of eosinophils (arrows, Fig. 8, I and J). Thus sections from insulin-treated diabetic antigen-challenged rats resembled sections from antigen-challenged rats.

Quantification of the number of eosinophils in the airways (Fig. 9A), and specifically in association with the airway nerves (Fig. 9B), confirmed that eosinophils were rarely seen in the airways or in association with protein gene product 9.5-immunoreactive airway nerves (in black). Scale bars, 50 µm in A, C, E, G, and I and 20 µm in B, D, F, H, and J.
the nerves of control and diabetic rats. In contrast, in antigen-challenged rats there was a significant rise in eosinophils in the airway wall and in association with the airway nerves compared with controls. This increase in eosinophils did not occur in the diabetic antigen-challenged rats, unless they were pretreated with insulin.

Mast cells were seen in the airways (Fig. 10A) and in association with the airway nerves (Fig. 10B) in all experimental groups. However, there were no significant differences in the numbers of mast cells in the airways or in association with the nerves among any of the groups of rats.

**DISCUSSION**

Intravenous ACh caused a dose-related bronchoconstriction in vagotomized animals that was not different between any of the groups of animals. These data demonstrate that neither antigen challenge nor diabetes alters postjunctional M₃ muscarinic receptor function. These data agree with previous reports that M₃ muscarinic receptor function is not altered by antigen challenge in guinea pigs (17) or by diabetes in rats (6). However, in nonvagotomized animals, intravenous methacholine caused bronchoconstriction, which was potentiated in antigen-challenged rats and attenuated in diabetic rats. These data confirm that antigen challenge results in vagal hyperresponsiveness (10) and that diabetes results in vagal hyporesponsiveness (6, 21) in animals and in humans. Antigen challenge of
diabetic rats did not cause vagally mediated airway hyperresponsiveness. This inhibition of antigen-induced vagal hyperresponsiveness after antigen challenge was dependent on decreased insulin in diabetic antigen-challenged rats, since vagal hyperresponsiveness was seen in diabetic antigen-challenged rats treated with insulin.

These data suggest that modulation of airway reactivity after diabetes and antigen challenge is due to changes in function of the parasympathetic nerves within the airways. Because neuronal inhibitory M₂ muscarinic receptors limit ACh release from parasympathetic nerves, changes in function of these receptors could explain changes in vagal reactivity.

In control rats the muscarinic agonist pilocarpine attenuated vagally induced bronchoconstriction in a dose-related manner by stimulating neuronal M₂ muscarinic receptors. This indicates normal function of the neuronal M₂ muscarinic receptor and confirms previous studies that have demonstrated the presence of functional neuronal M₂ muscarinic receptors in the rat (1, 6). In diabetic rats, pilocarpine inhibited vagally induced bronchoconstriction to a significantly greater degree than in normal rats, confirming increased function of neuronal M₂ muscarinic receptors in the lungs in the absence of insulin (6). The neuronal M₂ muscarinic receptors were dysfunctional in antigen-challenged rats, since pilocarpine did not inhibit vagally induced bronchoconstriction. This finding is in agreement with previous reports of neuronal M₂ muscarinic receptor dysfunction in antigen-challenged guinea pigs (11, 17) and in humans (5, 28) with asthma. Diabetes protected neuronal M₂ muscarinic receptor function from the effects of antigen challenge, since pilocarpine did inhibit vagally induced bronchoconstriction in these rats.

Loss of neuronal M₂ muscarinic receptor function after antigen challenge is due to eosinophil major basic protein (11, 12, 14, 15, 23, 24). Polyamionic compounds, such as heparin and poly-l-glutamate, neutralize cationic major basic protein in vivo, thereby acutely restoring neuronal M₂ muscarinic receptor function (15). In the current study, administration of heparin in the presence of pilocarpine reduced vagally induced bronchoconstriction in antigen-challenged rats to the level of control rats, demonstrating that neuronal M₂ muscarinic receptor dysfunction in antigen-challenged rats is also due to the presence of an endogenous, positively charged protein, such as eosinophil major basic protein.

In the airways of antigen-challenged rats, eosinophils were increased 100-fold as measured by bronchoalveolar lavage and 10-fold as measured by quantitative histology compared with control and diabetic rats. These data confirm previous studies showing significant eosinophilia in the lungs after antigen challenge in guinea pigs and in asthmatic humans (10). Specifically, there were more eosinophils associated with airway nerves in challenged rats than in controls, thus supporting the hypothesis that eosinophils and eosinophil major basic protein may be implicated in the decreased neuronal M₂ muscarinic receptor function after antigen challenge in rats.

In diabetic challenged rats, the neuronal M₂ muscarinic receptors were functioning normally and the number of eosinophils in airways was the same as in control rats. Preservation of functional neuronal M₂ muscarinic receptors in diabetic challenged rats may be due to a lack of eosinophil recruitment to the airway nerves of these animals. Insulin removed the protective effects that diabetes provided, since these animals were hyperresponsive to methacholine and demonstrated impaired neuronal M₂ muscarinic receptor function and airway eosinophilia.

Because the dose of insulin used in this study did not alter the hyperglycemia in the diabetic rats, the diabetes-induced changes in inflammatory cells and in M₂ muscarinic receptor function are likely to be due to an insulin deficiency rather than hyperglycemia. Insulin may suppress ketosis without affecting blood glucose levels. Because ketones were not measured, we cannot exclude the possibility that ketones may have prevented antigen-induced hyperreactivity in the diabetic animals. However, although insulin is required for the normal function of some inflammatory growth factors such as granulocyte-monocyte colony-stimulating factor (29), the role of ketones in eosinophilia is unknown. Thus insulin deficiency may preclude proper inflammatory cell production.

Impaired eosinophil recruitment to the airways may arise because of impaired chemotactant release from lymphocytes. In diabetic patients and diabetic rats, there appears to be a defect in interleukin-4 production (7, 13). Loss of interleukin-4 may prevent the proper differentiation of T lymphocytes bearing the CD4 antigen, a step considered important in coordinating the eosinophil response to antigen (30). Indirect evidence that there may be impaired T lymphocyte activation is suggested by the finding that in diabetic mice there is impaired production of a eosinophil chemotactant from T lymphocytes in response to infection with the parasite Schistosoma mansoni, an effect that can be reversed by insulin administration (27). Thus failure of T lymphocytes to produce eosinophil chemoattractants after antigen challenge could account for the impaired eosinophil influx seen in this study. Thus insulin may play a role in the recruitment of inflammatory cells by promoting their normal production or, indirectly, through promoting activation of T lymphocytes.

Another explanation for the findings reported here is that the allosteric interaction of eosinophil major basic protein with neuronal M₂ muscarinic receptors is impaired in diabetes. In diabetic tissues, agonists have increased binding affinity at the high- and low-affinity sites at M₂ muscarinic receptors (4, 6, 8, 25, 26). This altered affinity state for agonist binding may indicate that the allosteric site for major basic protein at the M₂ receptor is also altered. However, without eosinophils to bring the major basic protein to the receptor sites, it is unlikely that loss of an allosteric site is the only mechanism for M₂ receptor protection.

Numbers of mast cells in the airway wall and in association with airway nerves were also examined, since increased numbers of mast cells are seen in
association with rat enteric nerves and mast cell function is reported to be abnormal in diabetes (9). Although mast cells were frequently seen in association with airway nerves in our study, neither the total number of mast cells nor the number associated with airway nerves changed with diabetes or antigen challenge (Fig. 10).

In summary, neuronal M2 muscarinic receptors in rat lungs are dysfunctional after antigen challenge, resulting in vagal hyperresponsiveness. Eosinophils may be responsible for this loss of function in vivo, since eosinophils are associated with airway nerves and the anionic compound heparin reverses neuronal M2 muscarinic receptor dysfunction. The addition of diabetes to antigen-sensitized rats prevented eosinophil recruitment to the airway nerves and protected the function of the neuronal M2 muscarinic receptor from antigen challenge. Insulin restored the eosinophil influx into the lungs and removed the protection that diabetes provided to the neuronal M2 muscarinic receptors. Thus insulin appears to play a vital role in the development of airway inflammation, and since loss of neuronal M2 muscarinic receptor function leads to hyperresponsiveness, insulin also regulates hyperresponsiveness in the lungs.

In humans a mutual exclusion between diabetes and asthma has been reported in many epidemiologic studies (2, 22, 34). However, most of these studies were undertaken more than 20 years ago. Recently, it has been suggested that this inverse relationship between asthma and diabetes no longer exists (33). Stromberg et al. (33) suggest that modern high-quality insulin, along with better glycemic control, may explain the loss of mutual exclusion seen in past studies. If insulin is required for the development of eosinophilic inflammation, as the data presented in the current study suggest, then insulin treatment of diabetics may explain why it is possible for some modern-day diabetics also to have asthma.

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