Ozone-induced hyperresponsiveness and blockade of M₂ muscarinic receptors by eosinophil major basic protein

BETHANY L. YOST, GERALD J. GLEICH, AND ALLISON D. FRYER

1Department of Environmental Health Sciences, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland 21205; and 2Departments of Immunology and Medicine, Mayo Clinic, Rochester, Minnesota 55905

Yost, Bethany L., Gerald J. Gleich, and Allison D. Fryer. Ozone-induced hyperresponsiveness and blockade of M₂ muscarinic receptors by eosinophil major basic protein. J. Appl. Physiol. 87(4): 1272–1278, 1999.—Control of airway smooth muscle is provided by parasympathetic nerves that release acetylcholine onto M₂ muscarinic receptors. Acetylcholine release is limited by inhibitory M₂ muscarinic receptors. In antigen-challenged guinea pigs, hyperresponsiveness is due to blockade of neuronal M₂ receptors by eosinophil major basic protein (MBP). Because exposure of guinea pigs to ozone also causes M₂ dysfunction and airway hyperresponsiveness, the role of eosinophils in ozone-induced hyperresponsiveness was tested. Animals were exposed to filtered air or to 2 parts/million ozone for 4 h. Twenty-four hours later, the muscarinic agonist pilocarpine no longer inhibited vagally induced bronchoconstriction in ozone-exposed animals, indicating M₂ dysfunction. M₂ receptor function in ozone-exposed animals was protected by depletion of eosinophils with antibody to interleukin-5 and by pretreatment with antibody to guinea pig MBP. M₂ function was acutely restored by removal of MBP with heparin. Ozone-induced hyperresponsivity was also prevented by antibody to MBP and was reversed by heparin. These data show that loss of neuronal M₂ receptor function after ozone is due to release of eosinophil MBP.

neuronal receptors; hyperreactivity; asthma; inflammation

IN THE LUNGS, postganglionic parasympathetic nerves release acetylcholine onto M₂ muscarinic receptors on airway smooth muscle, thus causing contraction and bronchoconstriction (33–35). These parasympathetic nerves provide the dominant autonomic control of the airway smooth muscle, maintaining airway tone under resting conditions (31).

Neuronal M₂ muscarinic receptors inhibit release of acetylcholine from the parasympathetic nerves (12). Stimulation of the neuronal M₂ receptors with the agonist pilocarpine inhibits vagally induced bronchoconstriction by 70–80%. Conversely, blockade of the M₂ receptors with selective antagonists increases acetylcholine release (2) and increases vagally induced bronchoconstriction five- to eightfold (12). This demonstrates that the M₂ receptors exert a marked degree of control over acetylcholine release.

These neuronal M₂ receptors have been described in all species studied (5) including humans (29). They are dysfunctional in animal models of hyperreactivity (1, 11, 13, 29, 30, 36) and are also dysfunctional in some humans with asthma (1, 30). Decreased neuronal M₂ muscarinic receptor function results in increased release of acetylcholine and increased bronchoconstriction. Thus airway hyperresponsiveness could be mediated by loss of neuronal M₂ muscarinic receptor function.

Ozone-induced hyperresponsiveness is mediated by the parasympathetic nerves (17, 19, 20, 25, 37). Electrical stimulation of these nerves in guinea pigs and dogs produces significantly greater bronchoconstriction in animals exposed to ozone compared with air-exposed controls. In contrast, exposure to ozone does not affect dose-response curves to histamine, acetylcholine, or methacholine when the vagi are cut to eliminate any reflex component (17, 19, 20, 25, 37). Thus increases in vagally induced bronchoconstriction after ozone are due to increased release of acetylcholine and not to increased responsiveness of airway smooth muscle.

Exposure to ozone is associated with loss of neuronal M₂ muscarinic function and with an influx of inflammatory cells into the lung. Inflammatory cells are responsible for loss of neuronal M₂ receptor function and for hyperreactivity, because depletion of inflammatory cells with cytotoxic agents protects neuronal M₂ receptor function (15) and prevents hyperresponsiveness after ozone inhalation (32).

In antigen-challenged guinea pigs, loss of neuronal M₂ receptor function is mediated specifically by the recruitment of eosinophils to the airway nerves and release of eosinophil major basic protein (MBP). Eosinophil MBP is an antagonist for the neuronal M₂ receptors (21, 22). Depletion of eosinophils with an antibody to interleukin-5 (AbIL-5) (7), prevention of eosinophil migration into the lungs with an antibody to very-late-activation antigen-4 (9), and binding of eosinophil MBP with an antibody (AbMBP) (8), each protect neuronal M₂ muscarinic receptor function and prevent hyperresponsiveness in antigen-challenged guinea pigs. In addition, neutralization of eosinophil MBP in vivo with heparin acutely restores receptor function and reverses hyperreactivity (10). Thus, in antigen-challenged guinea pigs, hyperreactivity is mediated by eosinophil MBP and loss of neuronal M₂ muscarinic receptor function.

Viral infection of guinea pigs also causes loss of neuronal M₂ muscarinic receptor function (11). However, in contrast to the mechanism in antigen-challenged guinea pigs, eosinophils are probably not involved in this model, because heparin does not restore receptor function (14). The purpose of this study was to determine whether the loss of M₂ muscarinic receptor function and hyperreactivity after ozone are mediated by eosinophils.

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METHODS

Animals. Specific pathogen-free guinea pigs were used (all females, 350–400 g; Hilltop Lab Animals, Scottsdale, PA). All guinea pigs were shipped in filtered crates, housed in high-efficiency particulate-filtered air, and fed a normal diet (Prolab; Agway, Syracuse, NY). All animals were treated in accordance with the standards established by the US Animal Welfare Acts set forth in the National Institutes of Health guidelines and the Policy and Procedures Manual published by the Animal Care and Use Committee of the Johns Hopkins University School of Hygiene and Public Health.

Ozone exposure. Guinea pigs were placed in individual wire mesh cages within a 700-liter stainless steel exposure chamber with laminar airflow and were exposed to either ozone (2.0 parts/million (ppm)) or to filtered air for 4 h. Ozone was generated by an ultraviolet light generator (Orec, Glendale, CA) and was introduced into the chamber airflow at a rate of 2 l/min. Ozone concentrations within the chamber were monitored (model 1008AH, Dasibi Environmental, Glendale, CA), calibrated against a known ozone source, and recorded on a strip-chart recorder. The air supply within the chamber was replaced at a rate of 20 times/h. Control animals were exposed to filtered air under identical conditions.

Antibodies. Some of the guinea pigs were treated with either AbIL-5 (240 µg/kg ip; given 3 days before ozone exposure) or AbMBP (1 ml ip; given 2 h before ozone exposure).

Anesthesia and measurement of pulmonary inflation pressure (Ppi). At 24 h after ozone exposure, guinea pigs were anesthetized with urethan (1.5 g/kg ip). This dose of urethan produces a deep anesthesia that lasts 8–10 h (18), although most of the experiments described here were completed within 3 h.

The right carotid artery was cannulated to measure blood pressure with a transducer (DTX, Spectramed, Oxnard, CA). Heart rate was derived electronically from the pressure signal. The jugular veins were cannulated for the administration of drugs. Both vagus nerves were cut, and the distal ends were placed on platinum electrodes and bathed in a pool of liquid paraffin. Animals were tracheostomized, ventilated with a positive-pressure, constant-volume rodent ventilator (Harvard Apparatus, South Natick, MA) at a tidal volume of 1.0 ml/100 g body weight and 100 breaths/min. They were paralyzed with a constant infusion of succinylcholine (10 µg/kg/min) and diluted in 0.9% NaCl, except heparin and AbMBP (which were undiluted).

Ppi was measured at the trachea by using a pressure transducer (DTX, Spectramed). A positive pressure of 100–200 mmH2O was needed to adequately ventilate the animals. Signals were recorded on a polygraph (Grass Instrument, Quincy, MA).

Bronchoconstriction was measured as the increase in Ppi over the basal inflation pressure produced by the ventilator (6). The sensitivity of the method was increased by recording baseline Ppi on one channel, and increases in Ppi above this baseline were recorded on a separate channel at a higher sensitivity. This method provides a way to measure accurately increases in Ppi as small as 2–3 mmH2O above the baseline.

Measurement of vagally induced bronchoconstriction. All animals were pretreated with guanethidine (5 mg/kg iv) to deplete noradrenaline at least 25 min before the start of the experiment (4). Electrical stimulation of both vagus nerves produced bronchoconstriction (measured as an increase in Ppi) and bradycardia, which were due to release of acetylcholine onto muscarinic receptors, because they were abolished by atropine (1 mg/kg iv). Pulse duration (0.2 ms), train stimulus (5 s), and voltage (10 V) were kept constant, while frequency (1–25 Hz) was increased. The vagus nerves were stimulated at 2-min intervals. Heparin (2,000 U/kg iv) was administered to some guinea pigs, and vagally induced bronchoconstriction was measured 25 min later.

Measurement of neuronal M2 muscarinic receptor function. Pilocarpine is a muscarinic agonist with selectivity for neuronal over postjunctional receptors (24). Neuronal M2 muscarinic receptor function was measured by the ability of pilocarpine to inhibit vagally induced bronchoconstriction in a dose-related manner (12). The effect of pilocarpine is frequency dependent, being greater at 2 Hz than at higher frequencies (12). Thus all experiments that used pilocarpine were carried out at 2 Hz. Both vagus nerves were electrically stimulated (2 Hz, 0.2-ms pulse duration, and 22-s stimulus train) at 1-min intervals. The voltage was chosen within the range of 5–15 V to give an increase in Ppi of 15–25 mmH2O.

Once the vagally induced bronchoconstrictions were stable and consistent, increasing doses of pilocarpine (0.1–100 µg/kg iv) were administered. The effect of pilocarpine on vagally induced bronchoconstriction was measured as the ratio of bronchoconstriction in the presence of pilocarpine to bronchoconstriction in the absence of pilocarpine.

Measurement of M3 muscarinic receptor function on airway smooth muscle. The function of M3 muscarinic receptors on airway smooth muscle was tested by measuring the degree of bronchoconstriction induced by administration of methacholine (1–10 µg/kg iv). Guinea pigs were vagotomized to eliminate any possibility of a reflex component (3).

Bronchoalveolar lavage (BAL). At the end of each experiment, BAL was performed in situ via the tracheal cannula. The lungs were lavaged with 5 aliquots (10 ml each) of warm PBS. The recovered lavage fluid (40–45 ml) was centrifuged, the cells were resuspended in 10 ml of PBS, and total cells were counted by using a hemocytometer. Aliquots of the cell suspension were cytopsined onto glass slides, stained (Diff-Quik; Scientific Products, MacGaw Park, IL), and counted to obtain differential cell counts (38).

Antibodies and drugs. AbIL-5 (TRFK-5) was purchased from Pharmingen (San Diego, CA). Purified rabbit anti-guinea pig MBP (AbMBP) was the same reagent as described previously (26). Atropine, guanethidine, pilocarpine, methacholine, succinylcholine, and urethan were purchased from Sigma Chemical (St. Louis, MO). Heparin was purchased from Elkins-Sinn (Cherry Hill, NJ). All drugs were dissolved and diluted in 0.9% NaCl, except heparin and AbMBP (which were used undiluted).

Statistics. All data are expressed as means ± SE. Methacholine, frequency, and pilocarpine responses were analyzed by using two-way ANOVAs for repeated measures. Baseline heart rates, blood pressures, Ppi, and changes in Ppi (before pilocarpine administration), voltages used, and BAL were analyzed by ANOVA (Statview 4.5, Abacus Concepts, Berkeley, CA). A P value of 0.05 was considered significant.

RESULTS

Exposure to 2 ppm ozone for 4 h increased resting Ppi from 82 ± 10 mmH2O in air-exposed controls to 185 ± 26 mmH2O at 24 h after ozone. Neither pretreatment with AbIL-5 (183 ± 9 mmH2O), nor AbMBP (153 ± 6 mmH2O), nor administration of heparin (210 ± 6 mmH2O) affected this increased baseline inflation pressure. Resting heart rate (284 ± 6 beats/min) and blood pressure (62 ± 4 mmHg systolic; 33 ± 3 mmHg diastolic) were not affected by ozone or by any of the treatments.
Electrical stimulation of both vagus nerves (2 Hz, 0.2-ms pulse duration, 5–15 V, 44 pulses per train) produced bronchoconstriction (measured as an increase in Ppi) and bradycardia. Both of these responses to electrical stimulation were transient and were rapidly reversed after stimulation of the vagi was stopped. Both vagally induced bronchoconstriction and bradycardia were completely blocked by atropine (1 mg/kg iv). This indicates that these responses were mediated by the release of acetylcholine onto muscarinic receptors.

In the absence of pilocarpine, the voltages used to stimulate the vagus nerves were chosen within 5–15 V so that vagally induced bronchoconstriction was the same between groups. To obtain matched bronchoconstrictions, voltages in the lower end of this range were used in the ozone-exposed group, whereas voltages in the upper end of this range were used in the air-exposed group. However, the voltages between groups were not significantly different from each other.

The function of the neuronal M₂ muscarinic receptors was tested by using the agonist pilocarpine. In air-exposed guinea pigs, pilocarpine (1–100 µg/kg iv) inhibited vagally induced bronchoconstriction in a dose-dependent manner (Fig. 1). This demonstrates that the neuronal M₂ receptors were functional. The ability of pilocarpine to inhibit vagally induced bronchoconstriction was completely abolished in guinea pigs exposed to ozone (Fig. 1). However, in animals pretreated with AbIL-5 (Fig. 1) or AbMBP (Fig. 2) before ozone exposure, the response to pilocarpine was identical to the response in air-exposed control guinea pigs. Administration of intravenous heparin, 24 h after ozone exposure and 30 min before testing M₂ receptor function, completely restored the dose-response curve to pilocarpine in ozone-exposed guinea pigs (Fig. 3). The pilocarpine dose-response curves in ozone-exposed animals pretreated with AbIL-5 or AbMBP or posttreated with heparin were not significantly different from control.

Electrical stimulation of the vagus nerves (1–25 Hz) caused frequency-dependent bronchoconstriction (Fig. 4). Exposure to ozone significantly increased vagally induced bronchoconstriction (Fig. 4). However, in the guinea pigs pretreated with the AbMBP, this potentiation was reduced and was no longer significantly different from control. Heparin also significantly...
reduced vagally induced hyperresponsiveness after ozone (Fig. 5).

The response of the M3 receptors on airway smooth muscle was tested in vagotomized guinea pigs by measuring methacholine-induced bronchoconstriction. Neither ozone nor treatment with AbMBP or heparin altered methacholine-induced bronchoconstriction (Fig. 6).

Exposure of guinea pigs to ozone caused a small (not significant) increase in total cells recovered in BAL compared with controls (12.0 ± 1.5 vs. 16.2 ± 1.4 million cells in controls vs. ozone-exposed animals, respectively). In animals pretreated with AbIL-5 (16.0 ± 2.6 million cells), AbMBP (17.6 ± 2.1 million cells), or intravenous heparin (18.3 ± 6.5 million cells), there was no difference compared with ozone or control BAL. The small increase in total inflammatory cells is made up of significant increases in neutrophils and eosinophils (Fig. 7). The increase in eosinophils was inhibited only by AbIL-5 (Fig. 7), whereas the increase in neutrophils was not affected by AbIL-5, AbMBP, or heparin (Fig. 7). No treatment affected lymphocyte or macrophage numbers in lavage fluids.

DISCUSSION

In anesthetized guinea pigs, baseline heart rate and blood pressure were not significantly different between control and ozone-exposed animals. Neither administration of AbIL-5 or AbMBP before ozone nor administration of heparin after ozone affected baseline heart rate or blood pressure.

In the heart, a homogenous population of M2 muscarinic receptors (23) causes bradycardia when stimulated by acetylcholine. Bradycardia induced either by intravenous methacholine or by electrical stimulation of the vagus nerves was not different among control, ozone, or ozone-exposed guinea pigs treated with AbMBP, AbIL-5, or heparin. This indicates that the M2 muscarinic receptors in the heart were not altered by any of these treatments.

In contrast to the effects on the heart, exposure to ozone did increase baseline Ppi in vagotomized guinea pigs. However, this increase did not affect measurement of neuronal M2 muscarinic receptor function or of...
hyperreactivity. Treatments that restored the dose-response curve to pilocarpine or inhibited hyperreactivity (such as AbMBP, AbIL-5, or heparin) did not inhibit this increase. Furthermore, dose-response curves to the intravenous agonist methacholine, which were unchanged by exposure to ozone, were also not affected by this increase in baseline. Thus the ozone-induced increase in baseline Ppi did not contribute to hyperreactivity.

In the lungs, vagally induced bronchoconstriction was significantly potentiated 24 h after exposure to ozone (see Fig. 4). In contrast, bronchoconstriction induced by intravenous agonist methacholine, which were unchanged by exposure to ozone, were also not affected by this increase in baseline. Thus the ozone-induced increase in baseline Ppi did not contribute to hyperreactivity.

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function (8). Furthermore, AbMBP does not inhibit recruitment of eosinophils into the airways or to the nerves (8).

Additional support for the conclusion that MBP mediates loss of M2 function in the ozone-exposed animals comes from effects of heparin, which neutralizes cationic proteins such as MBP (10). The function of the neuronal M2 muscarinic receptors in ozone-exposed guinea pigs was acutely restored by the anionic compound heparin within 20 min of administration (Fig. 3). Thus dysfunction of the neuronal M2 receptors in vivo is caused by the presence of an endogenous, positively charged substance that inhibits M2 muscarinic receptor function. These data also suggest that the neuronal M2 muscarinic receptors have not been damaged by exposure to ozone but are blocked by an endogenous, cationic antagonist, such as MBP.

Methacholine-induced bronchoconstriction was not potentiated by ozone in vagotomized guinea pigs (see Fig. 6). This confirms that hyperreactivity after exposure to ozone is vagally mediated (25, 36, 37). Ozone hyperresponsiveness is mediated via loss of neuronal M2 muscarinic receptor function, because protecting M2 receptor function with AbMBP significantly inhibited vagally mediated hyperresponsiveness. In addition, restoring M2 muscarinic receptor function acutely with intravenous heparin simultaneously reversed hyperresponsiveness. This supports the conclusion that hyperreactivity is caused by blockade of M2 receptors with the cationic MBP. However, in these ozone-exposed guinea pigs, restoring neuronal M2 receptor function did not completely reverse hyperreactivity (see Figs. 4 and 5). This is in contrast to antigen-challenged animals, in which protection of neuronal M2 muscarinic receptor function did completely inhibit hyperreactivity (8, 9). Thus airway hyperreactivity in ozone-exposed guinea pigs is largely, but not completely, the direct result of antagonism of the inhibitory, neuronal M2 muscarinic receptors by eosinophil MBP.

Therefore, ozone-induced hyperresponsiveness requires the presence of eosinophils in the lungs. Nonasthmatic patients have few eosinophils in the lungs. This may explain why many nonasthmatic patients do not develop hyperresponsiveness after exposure to ozone. However, because asthma is associated with bronchial eosinophilia, exposure of asthmatic patients to ozone may result in degranulation of resident eosinophils in the lungs and the development of hyperreactivity, mediated by eosinophil MBP and loss of neuronal M2 receptor function.

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