Acidinium bromide abrogates allergen-induced hyperresponsiveness and reduces eosinophilia in murine model of airway inflammation

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Abstract

Airway hyperresponsiveness and inflammation characterize the airways of individuals with asthma and chronic obstructive pulmonary disease (COPD). Hence, therapeutic approaches that attenuate such manifestations may offer promise in the management of these diseases. In the present study, we investigated whether a novel long-acting cholinergic antagonist, acidinium bromide, modulates airway function and inflammation and hyperresponsiveness, the use of anticholinergics has been advocated to yield substantial therapeutic benefits in treatment of asthma and COPD. In human studies, the concomitant use of long-acting β2 adrenergic agonists and long-acting muscarinic receptor antagonists provides clinically relevant improvements in bronchodilation and patient symptoms compared to treatment with either agent alone (Tashkin et al., 2008; van Noord et al., 2006). In addition to restoring airway function, the combination of a long-acting muscarinic receptor antagonist with a long-acting β2 adrenergic agonist effectively decreases the rate of COPD exacerbations (Cazzola and Matera, 2008). In animal studies, such combinations substantially attenuate bronchoconstriction following a variety of challenges (Rossoni et al., 2007). Interestingly, even parameters independent of airway smooth muscle relaxation such as thromboxane A release were significantly altered by long-acting muscarinic receptor antagonists suggesting broader therapeutic benefits when used in conjunction with existing respiratory medications.

1. Introduction

Acetylcholine, a pivotal airway parasympathetic neurotransmitter, promotes airway smooth muscle shortening and mucus secretion in airways (Belmonte, 2005). As enhanced parasympathetic activity correlates with airway inflammation and hyperresponsiveness, the use of anticholinergics has been advocated to yield substantial therapeutic benefits in treatment of asthma and COPD. In human studies, the concomitant use of long-acting β2 adrenergic agonists and long-acting muscarinic receptor antagonists provides clinically relevant improvements in bronchodilation and patient symptoms compared to treatment with either agent alone (Tashkin et al., 2008; van Noord et al., 2006). In addition to restoring airway function, the combination of a long-acting muscarinic receptor antagonist with a long-acting β2 adrenergic agonist effectively decreases the rate of COPD exacerbations (Cazzola and Matera, 2008). In animal studies, such combinations substantially attenuate bronchoconstriction following a variety of challenges (Rossoni et al., 2007). Interestingly, even parameters independent of airway smooth muscle relaxation such as thromboxane A release were significantly altered by long-acting muscarinic receptor antagonists suggesting broader therapeutic benefits when used in conjunction with existing respiratory medications.

Among the five subtypes of muscarinic receptors (M1–M5), the M3 receptors are localized in airway smooth muscle tissue and mediate vagal and methacholine-induced bronchoconstrictor responses (Coulson and Fryer, 2003). Accordingly, bronchodilation occurs by selective antagonism of airway localized M3 receptors, while non-selective antagonism may also promote systemic blockade of M2 receptors inducing unwarranted cardiovascular effects (Barnes, 2004). Acidinium bromide, an inhaled cholinergic antagonist with sub-nano molar affinity for all muscarinic receptors, manifests a high kinetic selectivity for the M3 receptor (Gavalda et al., 2009b). In comparison to existing anti-muscarinic drugs including tiotropium, acidinium undergoes rapid hydrolysis in human plasma, resulting in low and transient systemic exposure, diminishing the potential for class-related systemic side effects. In the present study, using a well-characterized murine model of allergen-induced airway hyperresponsiveness and inflammation, we studied the effect of acidinium...
in modulating Aspergillus fumigatus (Af)-induced airway hyperresponsiveness. We also investigated the anti-inflammatory potential of aclidinium by measuring leukocyte numbers and cytokine levels in bronchoalveolar lavage fluid.

2. Materials and methods

2.1. Animals and sensitization protocol

Eight-12-week-old female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were housed under pathogen-free conditions. This study was approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Two cohorts of mice were evaluated: “Naive” mice received intranasal vehicle challenges with 21% glycerol in phosphate-buffered saline (PBS) and “Af-sensitized” mice injected intraperitoneally (i.p.) with 20 μg Af (Bayer Pharmaceuticals, Elkhart, IN) together with 20 mg alum (Imject Alum; Pierce, Rockford, IL) in 100 μl of PBS on Days 1 and 14, followed by intranasal challenge on Days 25, 26, and 27 with 25 μl of Af extract in PBS (12.5 mg in 21% glycerol, PBS) as described previously (Kurup et al., 1992). Af-sensitized and naive mice were anesthetized by inhalation of isoflurane, and 25 μl of Af extract or vehicle, respectively, was applied to the left naris. The studies were performed and all mice were sacrificed on Day 28, 24 h after their last intranasal treatment.

2.2. Aclidinium treatments

Subsets of Af-sensitized (Af+ AB) and naive mice (AB) were treated twice a day with aclidinium bromide (AB; 1 mg/ml) for three days (Days 25, 26, and 27) before assessing airway function and lung inflammation (Day 28). On each aclidinium treatment session, aclidinium was administered as an aerosol by an ultrasonic nebulizer in two 1-minute sessions with a 4-minute interval between each administration.

2.3. Assessment of airway responsiveness to methacholine

Airway responsiveness to methacholine was assessed using total lung resistance measurement by FlexiVent® as described previously (Haczku et al., 2001, 2000; Jain et al., 2008). Lung function tests were assessed 24 h after the Af or saline challenge. Bronchial reactivity to aerosolized methacholine was measured using the FlexiVent® system (SCIREQ, Montreal, Canada). Lung mechanics were studied in tracheostomized mice under anesthesia by intraperitoneal injection of ketamine and xylazine. Mice were ventilated with a computer-controlled small-animal ventilator (SCIREQ) with a rate of 150 breaths/min, and a positive end-expiratory pressure of 2 to 3 cm H2O. Once ventilated, mice were paralyzed with 0.8 mg/kg pancuronium bromide to block spontaneous breathing. The single-compartment model was fitted to these data by multiple linear regressions to calculate dynamic resistance and compliance of the airway.

2.4. Assessment of total protein, cytokine and immune cell in bronchoalveolar lavage fluid

Lungs were lavaged using 2 ml of sterile saline. The amount of liquid recovered after bronchoalveolar lavage averaged 1.75 ml. Total protein in cell-free bronchoalveolar lavage fluid was assayed by a BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL). Cytokine levels were determined from cell-free supernatant of the bronchoalveolar lavage fluid by ELISA using antibodies and recombinant cytokines from R&D Systems (San Diego, CA). Total and differential cell counts and ELISA analysis were performed as described previously (Kierstein et al., 2008).

2.5. Statistical analysis

To analyze the effect of different doses of methacholine on changes in lung resistance and dynamic compliance in the two groups, we used two-way ANOVA±Bonferroni adjustment. All values are represented as mean±S.E.M. Two means were considered significantly different when the P value was <0.05.

3. Results

3.1. Aclidinium reduces Af-induced airway hyperresponsiveness

The baseline lung resistance values in all groups (naive, Af, AB or Af + AB) after saline challenge were not significantly different from each other. As shown in Fig. 1, Af sensitization of mice induced a dose-dependent increase in methacholine-stimulated lung resistance with a peak resistance of 8.8±1.1 cm H2O s/ml at 20 mg/ml of methacholine. Aclidinium administration abrogated Af-induced increases in lung resistance at 5, 10 and 20 mg/ml of methacholine by 66±4%, 82±2% and 88±4%, respectively. As depicted in Fig. 2, with cumulative doses of methacholine, a dose-dependent decrease in dynamic compliance was also observed in both naive and Af-treated mice. In naive and Af-sensitized groups, aclidinium administration substantially (P<0.05, ANOVA) reversed methacholine-induced reduction in dynamic compliance. Naïve mice that received intranasal glycerol treatment alone showed no difference compared to non-sensitized, normal BALB/c mice in any of the study’s parameters.

3.2. Aclidinium attenuates Af-mediated changes in total proteins in bronchoalveolar lavage fluid

Total protein levels within bronchoalveolar lavage were assayed to index Af-induced lung injury and capillary leakage. As evidenced in Fig. 3, Af treatment increased the total amount of protein in bronchoalveolar lavage, and this effect was attenuated by aclidinium treatment by 63±18%.

3.3. Aclidinium diminishes Af-induced increases in bronchoalveolar lavage fluid eosinophil numbers

Quantitative and selective increases in bronchoalveolar lavage fluid-resident immune cell populations are important indicators of lung inflammation. Consistent with increased total protein content in bronchoalveolar lavage fluid, allergen sensitization significantly...
3.4. Aclidinium has no effect on Af-induced cytokine levels

As shown in Fig. 4, while Af treatment showed an eosinophil count of $10 \pm 1.3 \times 10^5$ cells, aclidinium treatment substantially diminished Af-induced eosinophilia to $4.3 \pm 0.3 \times 10^5$ cells (a decrease of $56 \pm 4\%$) with no significant effects on other immune cell types.

4. Discussion

Autoradiographic studies have shown M3 receptors on airway smooth muscle and epithelial cells of large and small human airways (Mak and Barnes, 1990). Additionally, M3 receptors are localized to enhanced cell numbers over naïve controls. Compared to naïve animals, assessment of constitutive cell populations in Af-sensitized groups showed a significant increase ($P<0.05$, ANOVA) in eosinophil numbers. As shown in Fig. 4, while Af treatment showed an eosinophil count of $10 \pm 1.3 \times 10^5$ cells, aclidinium treatment substantially diminished Af-induced eosinophilia to $4.3 \pm 0.3 \times 10^5$ cells (a decrease of $56 \pm 4\%$) with no significant effects on other immune cell types.

Following Af challenge, increased airway responsiveness and inflammation coincide with an increased number of eosinophils in the bronchoalveolar lavage fluid, and the depletion of eosinophils abrogates antigen-induced hyperreactivity (Hamelmann et al., 1997; Holgate et al., 1991). In our studies, aclidinium administration substantially inhibited Af-induced airway eosinophilia with no significant effects on the trafficking of other leukocytes. Our results support the conclusion from early studies where M3 receptor antagonists were shown to substantially diminish ovalbumin (OVA)-induced eosinophil influx in lung tissue (Bos et al., 2007). The precise mechanism by which aclidinium diminished Af-induced airway eosinophilia in our study remains unknown and could be similar to the role of atropine in inhibiting acetylcholine-mediated airway vascular leakage and plasma extravasations in guinea pig lungs (Cui et al., 2008).

In murine models of airway inflammation, prominent changes in total protein content in bronchoalveolar lavage fluid correlates with increased influx of leukocytes and vascular permeability (Haczku et al., 2001; Kleeberger and Hudak, 1992; Papouchado et al., 2001). As illustrated in Fig. 3, aclidinium treatment abrogated Af-induced increases in total protein content. Whether anticholinergics modulate airway responses by controlling eosinophil numbers or activation is unclear. While diminishing antigen-induced eosinophil numbers in sensitized guinea pigs, atropine pre-treatment potentiated M3 receptor-independent vagal hyperreactivity and enhanced eosinophil major basic protein presence. These studies imply that endothelial cells of the bronchial circulation and can mediate the vasodilator response to cholinergic stimulation (Wess, 2004). Functionally, bronchoconstrictor responses in lung tissue are primarily mediated via M3 receptors in airway smooth muscle, as confirmed in tissue preparations derived from M3R−/− knockout mice (Fisher et al., 2004). In this study, aerosolized aclidinium substantially inhibited Af-induced hyperresponsiveness in mice as evidenced by abrogation of methacholine-induced lung resistance and dynamic compliance. While aclidinium displays competitive antagonism by its association with both M2 and M3 receptor subtypes, AB dissociates faster from the M2 receptor than the M3 receptor, thus conferring a kinetic selectivity for the M3 subtype (M3 residence $t_1/2=4 \times M_2$ residence $t_1/2$) (Gavalda et al., 2009a). Studies in guinea pigs have shown that aclidinium has a fast onset of action ($t_{1/2}=6.8 \pm 1.5$ min, $t_{max}=35.9 \pm 8.2$ min) in reverting carbachol-induced tracheal contractions, long duration of action ($t_{1/2}=29$ h), and to undergo rapid hydrolysis in plasma ($t_{1/2}=2$ min) thus providing a favorable therapeutic index as a long-acting muscarinic receptor antagonist (Alberti et al., 2009).

Effective treatment of asthma requires control of airway hyperreactivity and eosinophilic inflammation. In the present study, aerosolized aclidinium significantly restored dynamic compliance and reduced bronchoalveolar lavage fluid protein content, likely via a competitive M3 receptor antagonism. However, the precise mechanism by which ACLIDinium diminished Af-induced airway eosinophilia in our study remains unknown and could be similar to the role of atropine in inhibiting acetylcholine-mediated airway vascular leakage and plasma extravasations in guinea pig lungs (Cui et al., 2008).
anticholinergics can regulate airway responses by modulating eosinophil activation (Verbout et al., 2007). Earlier reports also suggest that cholinergic agonists affect airway obstruction by modulating mucin secretion, smooth muscle proliferation and levels of inflammatory mediators (Cui et al., 2008; Gosens et al., 2006). Recent studies showed that acetylcholine-mediated activation of M3 receptors enhance chemoattractant factors that mediate leukocyte retention in airways (Profita et al., 2005). Similarly, stimulation of bovine tracheal strips with carbachol resulted in increased expression of IL-8, cyclooxygenase-1 and -2 at the mRNA level (Kanefsky et al., 2006). Plausibly, antagonism of M3 receptors by aclidinium could have diminished IL-4 or IL-6 levels in naïve or αF-sensitized animals. Data represents group mean ± S.E.M. Each cohort of naïve and αF consisted of 6 mice, and AB and αF + AB groups had 12 mice. Statistical analysis was performed by ANOVA ± Bonferroni adjustment. P<0.05 was considered to be significantly different.

Fig. 5. Aclidinium bromide does not alter αF-mediated cytokine levels in bronchoalveolar lavage fluid. Bronchoalveolar lavage fluid obtained from each mouse was assessed in triplicate for IL-4 (A) and IL-6 (B) by ELISA. AB treatment had no effect on either IL-4 or IL-6 levels in naïve or αF-sensitized animals. Data represents group mean ± S.E.M. Each cohort of naïve and αF consisted of 6 mice, and AB and αF + AB groups had 12 mice. Statistical analysis was performed by ANOVA ± Bonferroni adjustment. P<0.05 was considered to be significantly different.

IL-4 is a key cytokine in allergic inflammation secreted by varied cell types in the airway wall and bronchoalveolar lavage fluid. IL-4 orchestrates migration and residence of immune cells to inflammatory loci by induction of adhesion molecules in the pulmonary vasculature (Steinke and Borish, 2001). Earlier studies showed that in mice treated with anti-IL-4 antibody during allergen sensitization or the airway challenge of IL-4-deficient mice, both airway eosinophilia and airway hyperresponsiveness are modulated by the cytokine (Corry et al., 1996; Renz et al., 1995). Multiple external stimuli, including OVA, Af and ozone, enhance IL-6 secretions in murine airways. While disruption of IL-6-mediated responses by IL-6Rα-blocking antibody diminishes OVA-induced Th2 inflammation, others have shown that αF-mediated mucus secretions are substantially diminished in IL-6−/− mice, suggesting a prominent role for IL-6 in mediating/resolving an asthmatic phenotype (Doganci et al., 2005; Neveu et al., 2009). Evidence now shows that acetylcholine, its synthesizing enzyme choline acetyltransferase, and its functional receptors present in most airway resident cells and leukocytes can modulate physiological outcomes, including cytokine secretions, implying that M3 agonism or antagonism can regulate inflammatory mechanisms (Blanchet et al., 2007; Kanefsky et al., 2006; Klaproth et al., 1998; Racke and Matthiesen, 2004; Wessler and Kirkpatrick, 2001). However, aclidinium, in our studies, had little effect on αF-mediated IL-4 or IL-6 levels in bronchoalveolar lavage, implying that IL-4/IL-6-independent mechanisms likely modulate eosinophil localization and airway hyperresponsiveness. Our unexpected finding is consistent with other studies showing that airway hyperresponsiveness is regulated independent of airway inflammation and eosinophilia in animals deficient in IL-4Rα chain or in animals lacking signal transducer and activator of transcription (STAT)-6 (Kumar and Foster, 2002) or with studies where comparison of IL-6−/− or wild type mice showed no substantial differences in αF-induced airway eosinophils (Neveu et al., 2009).

Previously, clinical studies have shown that aclidinium improves lung function in patients with COPD. Our studies here show a potential therapeutic benefit for aclidinium bromide beyond a direct effect on bronchodilatation in effectively abrogating airway hyperresponsiveness and airway eosinophilia in allergen-induced airway hyperresponsiveness. These studies complement growing evidence for the benefits of long-acting anticholinergic agents as bronchodilators and potential anti-inflammatory agents in the treatment of asthma and COPD. Based on our studies, evaluating the role of aclidinium in mediating M3 receptor-driven outcomes in isolated human airway tissues and exploring the mechanisms by which aclidinium inhibits eosinophil accumulation in lungs would further delineate the mechanisms by which long-acting muscarinic receptor antagonists reduce bronchoconstriction and airway inflammation in asthma and COPD.

Conflict of interest

Jose Freire is an employee of Forest Research Institute, which supported this study. The authors alone are responsible for the content and writing of the paper.

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