

Chronic Exposure to Beta-Blockers Attenuates Inflammation and Mucin Content in a Murine Asthma Model

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Single-dose administration of beta-adrenoceptor agonists produces bronchodilation and inhibits airway hyperresponsiveness (AHR), and is the standard treatment for the acute relief of asthma. However, chronic repetitive administration of beta-adrenoceptor agonists may increase AHR, airway inflammation, and risk of death. Based upon the paradigm shift that occurred with the use of beta-blockers in congestive heart failure, we previously determined that chronic administration of beta-blockers decreased AHR in a murine model of asthma. To elucidate the mechanisms for the beneficial effects of beta-blockers, we examined the effects of chronic administration of several beta-adrenoceptor ligands in a murine model of allergic asthma. Administration of beta-blockers resulted in a reduction in total cell counts, eosinophils, and the cytokines IL-13, IL-10, IL-5, and TGF- β 1 in bronchoalveolar lavage, and attenuated epithelial mucin content and morphologic changes. The differences in mucin content also occurred if the beta-blockers were administered only during the ovalbumin challenge phase, but administration of beta-blockers for 7 days was not as effective as administration for 28 days. These results indicate that in a murine model of asthma, chronic administration of beta-blockers reduces inflammation and mucous metaplasia, cardinal features of asthma that may contribute to airflow obstruction and AHR. Similar to heart failure, our results provide a second disease model in which beta-blockers producing an acutely detrimental effect may provide a therapeutically beneficial effect with chronic administration.

Keywords: beta-blockers; beta-adrenoceptor; asthma; mucin; airway inflammation

Asthma mortality rates in the United States have risen by 60% in the last 25 years despite the widespread chronic therapeutic use of beta-adrenoceptor agonists (1). Recently, a large clinical trial using a long-acting beta-adrenoceptor agonist, salmeterol, was stopped due to an increased incidence of death and serious asthma-related events (2). Clearly there is a need for a different

CLINICAL RELEVANCE

This research may result in a paradigm shift in the treatment of asthma. This research demonstrates the importance that duration of beta-blocker therapy has on clinical and physiologic responses.

approach to chronic asthma therapy. Analogous to asthma, a different approach was needed in congestive heart failure (CHF) because the administration of beta-adrenoceptor agonists also produced acutely beneficial but chronically detrimental effects (3). Recently, drugs classified as beta-blockers (beta-adrenoceptor antagonists and inverse agonists) were introduced into therapy for CHF and have now become a part of first-line therapy (4). These drugs were once contraindicated in CHF because acute administration produced negative inotropic effects and decreased cardiac output (3). However, large clinical trials have shown that chronic administration improved cardiac output and decreased mortality in patients with CHF (4, 5). Notably, chronic administration of beta-blockers with partial agonist properties did not decrease mortality in CHF (6–8).

Presently, the administration of beta-blockers is contraindicated in asthma because their acute administration may cause increased airway resistance (9, 10). The effects of chronic administration of β -blockers in asthma have not been closely examined, perhaps because of the assumption that the chronic and acute effects of these drugs are similar (3, 11, 12). To determine if the assumption is incorrect, we previously investigated whether chronic administration of beta-blockers with inverse agonist properties could be useful in treating asthma (15). Beta-adrenoceptor inverse agonists are a subset of beta-blockers that, like all beta-blockers, can inhibit agonist-induced signaling, but inverse agonists can also inhibit signaling produced by constitutively active receptors (13). For the parameters that have been examined, agonists and inverse agonists produce the exact opposite effects (14). We therefore hypothesized that the reciprocal effects of agonists and inverse agonists may extend to their effect on signaling (14); specifically, that chronic agonist exposure can produce desensitization and inhibit signaling, while chronic treatment with inverse agonists may produce sensitization and enhance signaling. Our initial studies using a murine model of asthma showed that acute administration of beta-blockers with inverse agonist properties was detrimental and increased AHR, but chronic administration significantly

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decreased AHR (15). These data indicate the important role that duration of drug therapy can have on the observed physiologic or clinical response. Further support for our hypothesis is that as with CHF, chronic administration of a β -blocker with partial agonist properties did not decrease AHR in the murine model of asthma (6–8, 15).

In this study we have examined the effect of chronic treatment with beta-blockers on airway inflammation in an antigen-driven murine model of asthma. The inflammatory process in asthma involves the interaction between different immune cells such as lymphocytes and eosinophils, and parenchymal cells such as airway epithelial and smooth muscle cells. The nature of the interaction between these cells contributes to airflow obstruction, bronchoconstriction (including AHR), airway edema, and mucin hypersecretion (16, 17). Here we specifically measured the number of inflammatory cells and the level of the cytokines IL-13, IL-10, IL-5, transforming growth factor- β 1 (TGF- β 1), and regulated on activation, T cell expressed and secreted (RANTES) in bronchoalveolar lavage (BAL) fluid, and determined the degree of mucous metaplasia by airway epithelial cells.

MATERIALS AND METHODS

Mice

Six- to twelve-week old BALB/cJ (male) mice (Jackson Animal Laboratory, Bar Harbor, ME) and C57BL/6J (male and female) mice (Harlan Sprague Dawley, Indianapolis, IN) were housed under specific pathogen-free conditions in accordance with the Institutional Animal Care and Use Committee of the University of Houston and the M.D. Anderson Cancer Center, respectively.

Animal Sensitization and Challenge

In the

65 mg/ml) BALB/cJ mice, and repeated once. The total leukocyte count in the BAL was determined using a Coulter counter (Beckman Coulter, Fullerton, CA). Cytospins were prepared from 200 μ l of BAL at 500 rpm for 5 minutes and stained with Hema-3 for enumeration of cell types. Eosinophils, monocytes, lymphocytes, or neutrophils were identified by standard morphologic criteria. At least 200 cells were counted from cytospin preparations. BAL was also obtained as described above through the tracheal cannula in euthanized C57BL/6J mice. The total leukocyte count was determined using a hemacytometer, and cell populations were determined by cyto centrifugation of 300 μ l of BAL using a Cytospin 4 (Thermo Electron Corporation, Waltham, MA) at 2,000 rpm for 5 minutes, followed by Wright-Giemsa staining.

Histochemistry

For staining with periodic acid fluorescent Schiff's (PAFS) reagent to examine intracellular mucin, lungs were fixed with 4% paraformaldehyde in PBS (pH 7.0) infused through a tracheal cannula at room temperature, then removed from the thoracic cavity and further fixed overnight at 4°C, embedded in paraffin, sectioned, and stained for light microscopy as previously described (19).

Fluorescence Microscopy

For the quantification of mucin, PAFS-stained slides were examined under a $\times 40$ objective. Images of 10 fields from the axial bronchi were captured, and camera settings were managed using MagnaFire 2.1 (Optronics, Goleta, CA) (19). The volume density of mucin in the airway epithelium was then measured using ImagePro Plus and calculated as previously described (19). Images were acquired before any measurements and analyzed by blinded investigators.

Immunohistochemistry

Immunohistochemical staining was performed on 5- μ m paraffinized sections of C57BL/6J mice lung tissue after paraformaldehyde (Sigma) fixation. Sections dewaxed with xylene (Sigma-Aldrich) were placed in 100% ethanol for 2 \times 5 minutes, followed by 3% H₂O₂ (Sigma) in methanol for 10 minutes to quench endogenous peroxidase activity, and then fully rehydrated. Antigen retrieval was performed in citrate buffer 10 mM, pH 6.0 for 10 minutes at 100°C. Goat serum (Vector Laboratories, Burlingame, CA) diluted in PBS was used to block non-specific binding sites. For Muc5ac, the primary antibody used was chicken polyclonal anti-Muc5ac (20) at a 1:2,000 dilution. After 1 hour of incubation at 23°C, the biotinylated secondary antibody goat anti-chicken (Invitrogen, Carlsbad, CA) was applied at 1:500 for 30 minutes at 23°C. For Muc5b, primary antibody used was rabbit anti-Muc5B (from C.E. and M.K.) at 1:10,000 for 1 hour at 23°C followed by incubation with secondary antibody goat anti-rabbit-horseradish peroxidase at 1:200 for 30 minutes at 23°C. The Muc5b antibody was raised against a 15-aa synthetic peptide corresponding to a sequence that was repeated six times in the tandem repeats of Muc5b between aa1560 and aa3370. Bound antibody was visualized using the Vectastain ABC staining kit (Vector Laboratories). Slides were examined under a $\times 60$ objective.

RNA Isolation and RT-PCR

Lungs were removed from killed mice, washed in PBS, and snap-frozen in liquid nitrogen. For RNA extraction, tissues were homogenized in TRIZOL reagent (Invitrogen). Five micrograms of total RNA was reverse transcribed using random 9-mers as complementary DNA (cDNA) primers in a 20- μ l reaction. For quantitative PCR, we used TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA) and TaqMan Gene Expression Assays (Applied Biosystems) containing a mixture of unlabeled PCR primers and FAM dye-labeled TaqMan probes for Muc5ac (Assay Mm 01,276,725 \times g1) and Muc5b (Assay Mm 00,466,376 m1), and VIC dye-labeled TaqMan probe for 18S (Assay 4319413E). Quantitative PCR was performed using 100 ng of cDNA in a 20- μ l reaction, using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). A standard curve was generated by running in parallel synthetic single stranded oligonucleotide templates for each transcript at dilutions of 10 to 0.001nM. The results were expressed as number of mucin gene copies per 18S copies.

Cytokine and Chemokine Levels

The levels of IL-13, IL-10, IL-5, TGF- β 1, and RANTES in BAL obtained 24 hours after the last antigen challenge were measured by Pierce Searchlight (Woburn, MA) using multiplex enzyme-linked immunosorbent assay. The samples were provided to Pierce Searchlight in blinded fashion.

Statistical Analysis

Quantitative data are presented as mean \pm SEM (expressed as the percent standard error of the mean). Statistical analysis for multiple groups was performed using one-way ANOVA followed by Dunnett's multicomparison test. Unpaired *t* test was performed for analysis of two groups (Prism, GraphPad software, CA). *P* < 0.05 was considered statistically significant.

RESULTS

Chronic Administration of Beta-Blockers Reduces Airway Eosinophilia and Cytokine Levels

The total cell count in BAL was greatly increased in antigen-challenged immunized BALB/cJ mice compared with saline-challenged mice (Figure 2A). Chronic administration of the selective β_2 -adrenoceptor antagonist ICI 118,551 (28 d), or the nonselective β -adrenoceptor antagonist nadolol (13) (28 d), significantly reduced total cell counts in antigen-challenged mice (Figure 2A). BAL eosinophil numbers were also greatly increased in antigen-challenged mice compared with saline-challenged mice (Figure 2B), and again, chronic administration with ICI 118,551 or nadolol significantly reduced BAL eosinophil numbers (Figure 2B). A similar effect was observed by a second laboratory using a different strain of mice (C57BL/6J instead of BALB/cJ and a different sensitization and challenge protocol; see MATERIALS AND METHODS), with total cell counts reduced by approximately 80% (from $1.4 \times 10^5 \pm 6.5\%$ to $0.3 \times 10^5 \pm 11\%$) and eosinophils reduced by approximately 90% (from $1.0 \times 10^5 \pm 9.6\%$ to $0.1 \times 10^5 \pm 38.6\%$) (*n* = 3, *P* < 0.05 for each comparison) after chronic administration of ICI 118,551

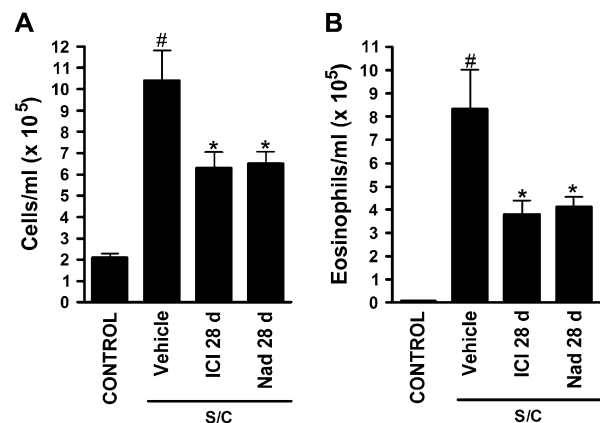


Figure 2. Effects of chronic administration of beta-blockers on cell count and eosinophils in bronchoalveolar lavage (BAL) of antigen-sensitized and -challenged BALB/cJ mice. (A) Total cell count and (B) eosinophil numbers in BAL from saline-challenged mice (CONTROL), antigen-challenged mice (S/C) administered vehicle, the selective β_2 -adrenoceptor antagonist ICI 118,551 for 28 days (ICI 28 d), or the nonselective β -adrenoceptor antagonist nadolol for 28 days (Nad 28 d) (Figure 1, protocol 1). BAL was collected 24 hours after the last challenge. Values are the means \pm SEM of data from 6 to 12 mice in each group (*n* = 6–12). [#]*P* < 0.05, significantly different compared with saline-challenged mice; ^{*}*P* < 0.05, significantly different compared with antigen-challenged mice.

(14 d). These findings suggest that chronic administration of β -blockers modifies eosinophilic airway inflammation in a murine model of asthma. Antigen-challenged mice also had elevated levels of the cytokines IL-13, IL-10, IL-5, TGF- β 1, and RANTES in BAL compared with saline-challenged mice (Figure 3). Consistent with the changes in cellular inflammation, chronic administration of nadolol resulted in a reduction in the levels of the cytokines IL-13, IL-10, IL-5, and TGF- β 1 (Figure 3).

Chronic Administration of Beta-Blockers Attenuates Airway Epithelial Cell Mucin Production

To determine whether chronic administration of beta-blockers affects mucin content, sections from the axial bronchi of experimental mice were assessed using PAFS staining. Minimal intracellular mucin was observed in saline-challenged mice, whereas in antigen-challenged mice there was increased mucin content and airway epithelial cell height (mucous metaplasia) (Figure 4). Within the antigen-challenged mouse group, chronic administration of the β_2 -adrenoceptor agonist salbutamol had no significant effect on mucous metaplasia (Figure 4). However, chronic administration of ICI 118,551 or nadolol for 28 days decreased mucin content and partially reversed the changes in airway epithelial cell morphology (Figure 4). A similar effect was observed in antigen-challenged C57BL/6J mice, with mucin volume density reduced by more than 90% from $14.7 \text{ nl/mm}^2 \pm 6.3\%$ to $1.2 \text{ nl/mm}^2 \pm 55.6\%$ ($n = 3$, $P < 0.05$) after chronic administration of ICI 118,551 (14 d).

Chronic Administration of Beta-Blockers during the Challenge Phase Alone Reduces Airway Inflammation and Mucin Production

In our initial experiments, the 28-day administration of beta-adrenoceptor ligands (salbutamol, ICI 118,551, or nadolol) occurred during both the OVA sensitization and challenge phases (Figure 1, *protocol 1*). To determine which phase is critically affected by beta-blockers, a second set of experiments was performed in which exposure to the beta-adrenoceptor

ligands was begun after completion of the sensitization process (Figure 1, *protocol 2*). Administration of beta-blockers for 28 days beginning after the completion of sensitization produced similar reductions in mucous metaplasia (compare Figure 4B, *protocols 1* and 2), thus ruling out interference by the beta-adrenoceptor ligands with the sensitization process as a cause of the observed decreases in mucous metaplasia. Furthermore, the administration of ICI 118,551 for 7 days beginning after the completion of sensitization reduced BAL total cell counts from $10.4 \times 10^5 \pm 13.6\%$ to $5.4 \times 10^5 \pm 16.6\%$ ($n = 5-12$, $P < 0.05$), a reduction very similar in degree to that observed with the administration of beta-blockers for 28 days during both sensitization and challenge (Figure 2). Also, the results with C57BL/6J mice, in which exposure to ICI 118,551 occurred after completion of the sensitization process (Figure 1, *protocol 3*), parallel the results observed in the BALB/cJ mice. Surprisingly, 7 days of beta-blocker during the challenge phase alone did not reduce mucous metaplasia to the same extent as 28 days of beta-blocker during the challenge phase (Figure 4B, *protocol 2*). Decreased mucin production was confirmed by immunohistochemical staining in C57BL/6J mice (Figure 5A) and by quantification of Muc5ac transcripts in both C57BL/6J and BALB/cJ mice (Figure 5B). Muc5ac is the predominant mucin upregulated in airway epithelial cells in patients with asthma and in the ovalbumin mouse models of asthma (21, 22). The results show that Muc5ac staining and transcripts are markedly increased in antigen-challenged mice compared with saline-challenged mice, but both are decreased in antigen-challenged mice treated with ICI 118,551 or nadolol (28 d) (Figure 5). We also performed immunohistochemical staining (Figure 5A) and quantification of transcripts for Muc5b (Figure 5B). This mucin does not change substantially in antigen-induced mucous metaplasia (21, 22), and, as expected, antigen sensitization and challenge produced only a small change in Muc5b. By immunohistochemical staining, Muc5b was apparent at the apical pole of epithelial cells in saline-challenged mice, became redistributed throughout the distended cytoplasm in antigen-challenged mice, and did not change appreciably in antigen-challenged mice treated with ICI 118,551 (Figure 5).

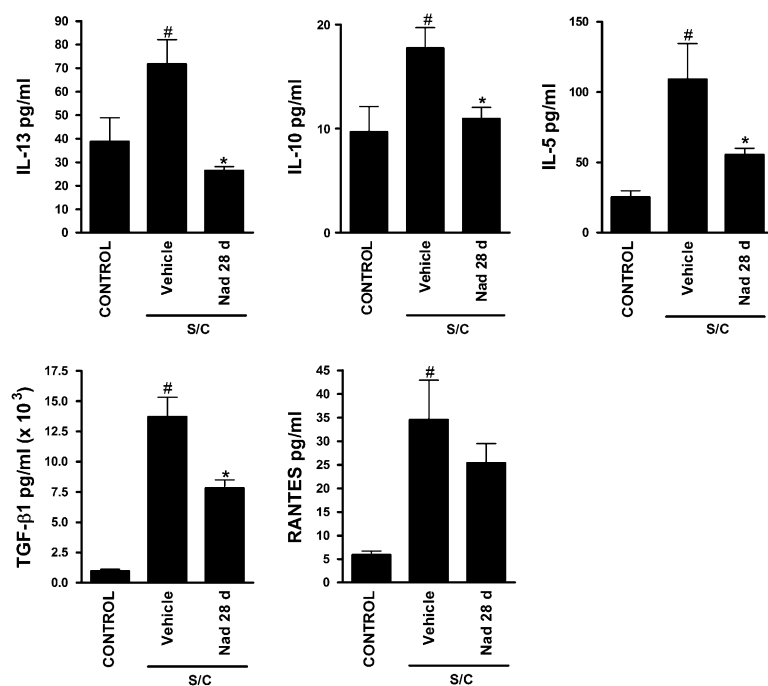


Figure 3. Effects of chronic administration of nadolol on cytokines levels in the BAL of BALB/cJ mice. The concentration of IL-13, IL-10, IL-5, TGF- β 1, and RANTES was measured by multiplex enzyme-linked immunosorbent assay analysis of BAL harvested 24 hours after the last challenge from saline-challenged mice (CONTROL), antigen-challenged mice (S/C) administered vehicle or nadolol for 28 days (Nad 28 d) (Figure 1, *protocol 2*). Values are the means \pm SEM of data from seven to eight mice in each group ($n = 7-8$). # $P < 0.05$, significantly different compared with saline-challenged mice; * $P < 0.05$, significantly different compared with antigen-challenged mice.

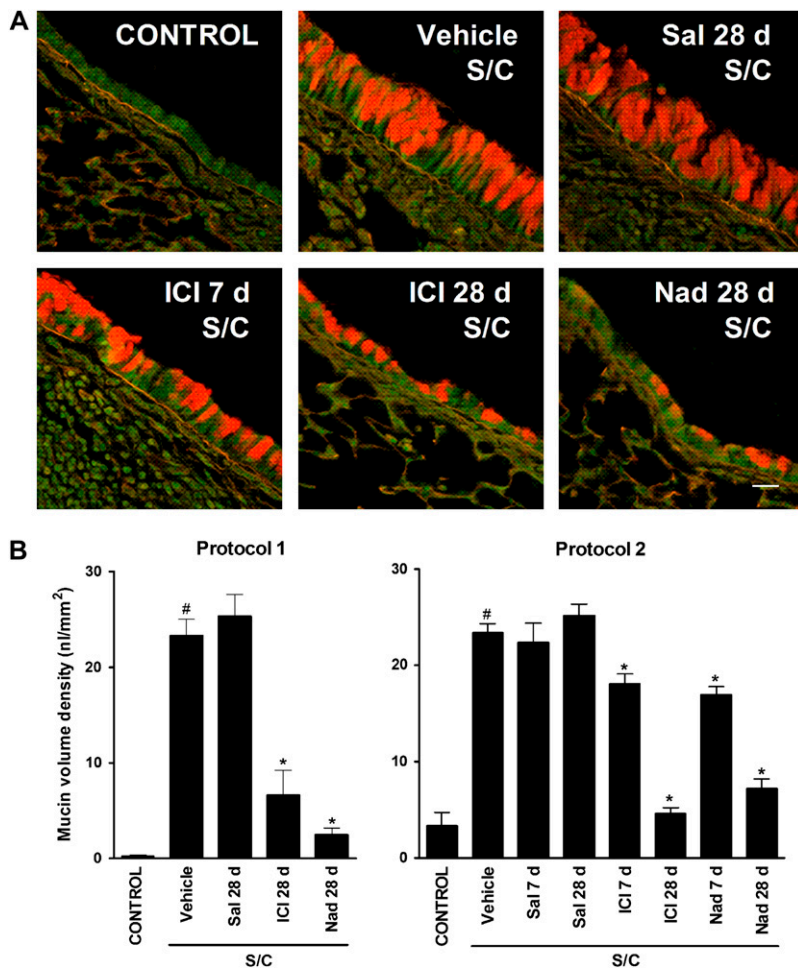


Figure 4. Effects of chronic administration of beta-adrenoceptor ligands on mucin content in the airway epithelium. Mucin content in the airway epithelia of BALB/c mice was measured using periodic acid fluorescent Schiff from (A) saline-challenged mice (CONTROL), antigen-challenged mice (S/C) administered vehicle, the beta₂-adrenoceptor agonist salbutamol for 28 days (Sal 28 d), the selective beta₂-adrenoceptor antagonist ICI 118,551 for 7 or 28 days (ICI 7 or 28 d), or the nonselective beta-adrenoceptor antagonist nadolol for 28 days (Nad 28 d) (Figure 1, protocol 1). Scale bar, 20 μm. (B) Morphometric quantification of the mucin volume density was assessed from the various groups (left panel using protocol 1 of Figure 1). A second set of experiments was performed to assess the effect of beta-adrenoceptor ligands administration during the challenge phase only (right panel using protocol 2 of Figure 1). Values are the means ± SEM of data from 3 to 10 mice in each group ($n = 3-10$). # $P < 0.05$, significantly different compared with saline-challenged mice; * $P < 0.05$, significantly different compared with antigen-challenged mice.

DISCUSSION

Based on the paradigm shift that occurred in the treatment of heart failure with regard to beta-adrenoceptor drugs, we hypothesized that chronic beta-blocker treatment may be beneficial in asthma (12, 15). Here we report that chronic treatment with “beta-blockers,” a class of drugs currently contraindicated in asthma, reduces airway inflammation in a murine model of asthma. A characteristic feature of asthma is the airway infiltration by inflammatory cells such as lymphocytes and eosinophils (16, 17). These cells produce a wide range of inflammatory mediators and cytotoxic proteins that are responsible for the perpetuation of airway inflammation (16, 17). Our results show that chronic treatment with nadolol or ICI 118,551 reduced the inflammatory cells in the BAL of antigen-challenged mice (Figure 2), and nadolol also reduced the levels of the cytokines IL-13, IL-10, IL-5, and TGF-β1 (Figure 3). The approximately 50% reduction in eosinophils in BAL was comparable to what we had previously reported, although in that study the reduction did not reach statistical significance ($P = 0.07$) (15). The effect of beta-blockers on BAL cellularity was not confined to one strain of mice, since it was observed in both BALB/cJ and C57BL/6J mice. The results with C57BL/6J mice were performed by another laboratory using a slightly different sensitization and challenge protocol. Thus the effect of beta-blockers appears to be robust and relatively insensitive to experimental conditions.

The reduction in inflammatory cells and cytokines caused by chronic administration of beta-blockers suggested that target effects of airway inflammation might also be reduced by this

drug treatment. We therefore asked whether chronic administration of beta-blockers affected airway epithelial cell morphology and mucin content (17). The airway epithelium is a mechanical barrier that protects the underlying tissue from the external environment (23), but also shows great plasticity in structure and function (24–26). In asthma, the airway epithelium contains increased numbers of goblet cells filled with mucus, which contributes significantly to airflow obstruction and asthma morbidity (24, 26–28). At a molecular level, up-regulation of Muc5ac gene expression is the central event in mucous metaplasia (21). Chronic treatment with beta-blockers produced a marked time-dependent decrease in goblet cell numbers and mucin content of the airway epithelium (Figures 4 and 5). The effect of nadolol showed selectivity for Muc5ac staining and mRNA, as Muc5b, a polymeric mucin not substantially involved in the antigen-induced mucous metaplasia, was not affected by ICI 118,551 or nadolol administration (Figure 5) (22). The reduction in mucous metaplasia was not due to interference with the sensitization process, as the results were the same when treatment was begun after the sensitization process was complete (Figure 4, and the reduction in mucous metaplasia observed in the C57BL/6J mice). Furthermore, there was no significant change in serum IgE levels after chronic nadolol treatment (data not shown), which also suggests a lack of interference with the sensitization process.

The fact that the principal effect of beta-blockers is exerted during the challenge rather than the sensitization phase of our asthma models, and that the reduction in mucous metaplasia from beta-blockade is disproportionate to the reduction in lung inflammatory cells, suggests that beta-blockers exert an impor-

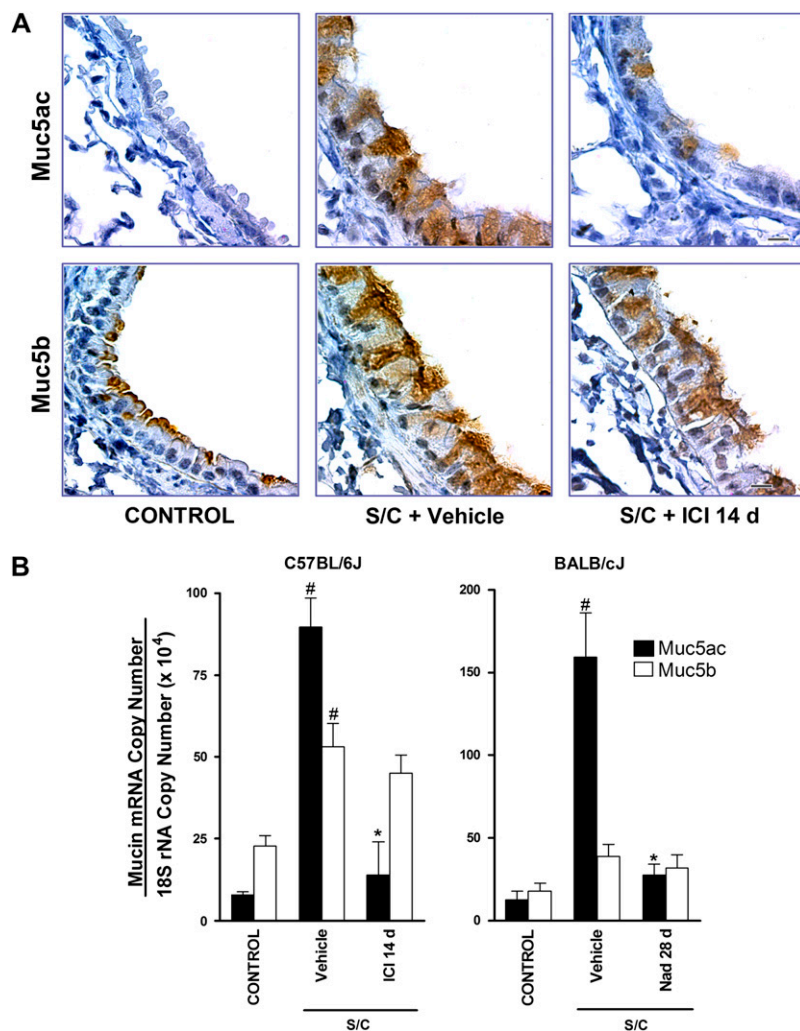


Figure 5. Effect of chronic administration of beta-blockers on Muc5ac and Muc5b expression. (A) Muc5ac and Muc5b glycoprotein expression was assessed in C57BL/6 mice using immunohistochemical staining of saline-challenged mice (CONTROL), and antigen-challenged mice administered vehicle (S/C + vehicle) or ICI 118,551 for 14 days (S/C + ICI 14 d) (Figure 1, protocol 3). Scale bar, 10 μ m. (B) Muc5ac and Muc5b transcripts were quantified by using quantitative PCR of RNA from the lungs of both C57BL/6 (left panel) and BALB/cJ (right panel) mice. Saline-challenged mice (CONTROL), and antigen-challenged mice (S/C) were administered vehicle or ICI 118,551 for 14 days (ICI 14 d) (Figure 1, protocol 3) or nadolol for 28 days (Nad 28 d) (Figure 1, protocol 1). Values are the means \pm SEM of data from three to five mice in each group ($n = 3-5$). [#] $P < 0.05$, significantly different compared with saline-challenged mice; ^{*} $P < 0.05$ significantly different compared with antigen-challenged mice.

tant effect on the epithelium itself. Supporting this hypothesis is the fact that chronic exposure to beta-blockers has been shown to produce a marked increase in beta₂-adrenoceptor numbers primarily in airway epithelial cells (29), and that overexpression of the beta₂-adrenoceptors in the airway epithelium reduces AHR (30). In our murine model of asthma, AHR reduction does parallel the reduction in BAL cellularity and also the mucin data to an extent. However, there are also differences. In experiments run in parallel to these but published separately (29), the observed reduction in AHR is maximal after 7 days of treatment with a beta-blocker (29) and stays reduced to the same extent at 28 days, thus following a time course similar to the cells and eosinophils in BAL, while the mucous metaplasia, though significantly reduced at 7 days, continues to decline further with 28 days of beta-blocker treatment. Comparing the effects of 7 days versus 28 days of beta-blocker treatment suggests that the reduction in mucous metaplasia from beta-blockade does not reach steady state after 7 days and that there are complex effects on epithelial cell signaling (24, 26). Mucous metaplasia is a cardinal feature of the asthma phenotype that is thought to play a central role in death from asthma (24, 25, 31). Recent studies also suggest that mucin hypersecretion can contribute significantly to AHR (27, 32, 33). Thus, the finding that chronic administration of beta-blockers reduces mucin content in airway epithelial cells suggests a possible identification of a cell type involved in the decreased AHR that we previously observed (15).

Together, these observations of the anti-inflammatory effect of beta-blockers in a murine model of asthma suggest that individuals with asthma may benefit from treatment with such drugs given their ability to reduce some indices of airway inflammation and to further reduce mucous metaplasia. Indeed, in a small (10 patients) clinical trial, treatment of subjects with mild asthma with nadolol for 9 weeks resulted in a dose-dependent increase in the concentration of methacholine required to produce a 20% fall in forced expiratory volume at 1 second (PC₂₀ methacholine value) (34).

In addition to investigating the differences we observed on mucous metaplasia between 28 and 7 days of treatment, future studies will explore differences in the changes produced by nadolol and other beta-blockers. So far, different effects on airway resistance have been observed for various beta-blockers (15), and whether all beta-blockers confer equal benefit in asthma therapy would be a subject of importance. Within the limitations of extrapolating from a murine model of asthma, these results provide a second disease model (besides heart failure) in which compounds producing an acutely detrimental effect may provide a therapeutically beneficial effect with chronic administration, and indicate that the chronic effect of drugs cannot be predicted from their acute effects (11, 12). Furthermore, similar to the paradigm shift in the treatment of heart failure, our current results with beta-blockers could potentially lead to another paradigm shift in the treatment of another important disease, asthma.

Conflict of Interest Statement: L.P.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. O.O. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.P. is a shareholder in Inverseon, Inc. J.M.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Z.A.-A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.B.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.J.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.J.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.F.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.A.B. is scientific founder and a shareholder of Inverseon, Inc.

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