Inhaled budesonide inhibits OVA-induced airway narrowing, inflammation, and cys-LT synthesis in BN rats

LIJING XU, RONALD OLVENSTEIN, JAMES G. MARTIN, AND WILLIAM S. POWELL
Meakins-Christie Laboratories, McGill University, Montreal, Quebec, Canada H2X 2P2

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The beneficial effects of glucocorticoids in asthmatic subjects may also be due in part to inhibitory effects on the synthesis of cysteinyl-LTs (cys-LTs), which are potent stimulators of airway smooth contraction (9, 35, 41), vascular leakage (8), and mucus secretion (23). Studies with inhibitors of LT synthesis (42) and LTD4 antagonists (32, 37) have clearly established the critical role of cys-LTs in asthma. Therapeutic intervention aimed at blocking the effects of these substances may be an important adjunct to inhaled glucocorticoids in the management of this disease (11, 19, 36).

The initial step in the synthesis of cys-LTs is the release of arachidonic acid from membrane lipids by phospholipase A2. Glucocorticoids block the increased expression of both the cytosolic, calcium-dependent (14, 20) and secreted (33) forms of this enzyme by interleukin-1 and tumor necrosis factor. Moreover, dexamethasone has been reported to strongly inhibit the retinoic acid-induced enhancement in LTC4 synthesis by rat basophilic leukemia cells (16). On the other hand, dexamethasone was found to enhance the expression of 5-lipoxygenase activating protein in neutrophils (27) and both 5-lipoxygenase activating protein and 5-lipoxygenase in monocytes (31). Glucocorticoids were also reported to increase the production of LTA4 by neutrophils from patients with rheumatoid arthritis (38). Despite the stimulatory and inhibitory effects of glucocorticoids on individual cell types, in vivo studies have failed to show any effects of these substances on the cys-LT levels in urine of both healthy (22, 34) and atopic asthmatic (26) human subjects. However, we recently demonstrated that systemic treatment of BN rats with dexamethasone (300 μg/kg ip 14 and 2 h before antigen challenge) blocked the antigen-induced increase in biliary cys-LT levels by 80% (28). This was accompanied by an ~60% reduction in the early airway response to antigen and elimination of the late response (28).

In view of widespread use of topical glucocorticoids in the treatment of asthma, it was important to determine whether topical, as well as systemic, glucocorticoids could inhibit the synthesis of cys-LTs in the BN rat animal model. To answer this question, we chose the potent nonhalogenated synthetic steroid budes-
Budesonide, which is retained well by the lung and has a long duration of action, possibly due in part to the reversible formation of lipophilic fatty acid esters (25). Budesonide has been reported to inhibit both early and late airway responses to antigen challenge (2) and to reduce the numbers of eosinophils and mast cells in the lungs of asthmatic subjects (18). We found that topical budesonide strongly inhibited the late airway response to antigen in BN rats and that this was associated with a reduction in both the in vivo production of cysteinyl leukotrienes and the numbers of pulmonary macrophages recovered in bronchoalveolar lavage (BAL) fluid.

METHODS

Animals. Highly inbred male BN rats, 7–9 wk old, were obtained from Harlan Sprague-Dawley (Walkerville, MD). Active sensitization was performed by subcutaneous injection of 1 mL of saline containing 1 mg of ovalbumin (OVA; grade V, Sigma Immunochemicals, St. Louis, MO) and 3.48 mg of aluminum hydroxide (EM Industries, Hawthorne, NY). At the same time, 0.3 mL of Bordetella pertussis vaccine containing 2 × 10^10 heat-killed organisms was given intraperitoneally as an adjuvant. Animals were studied 14–21 days after sensitization.

Study groups. Two groups of rats (n = 6 for each group) were lightly anesthetized with xylazine (7 mg/kg) and pentobarbital (30 mg/kg ip) before endotracheal intubation. Each rat then received either saline or 2.5 mg/kg budesonide (0.25 mg/ml) by aerosol over a period of 5 min using a disposable Hudson nebulizer (Hudson, Temecula, CA) at a flow rate of 8 l/min and an output of 0.15 ml/min. Seventeen hours later, the animals were again anesthetized, and these treatments were repeated. After a further hour, each of the rats was challenged with aerosolized 5% OVA in saline for 5 min. The experimental protocol is illustrated in Fig. 1.

Surgical procedures. Before the second treatment with budesonide or saline, the rats were anesthetized with urethane (1 g/kg ip, 50% wt/vol). After blind orotracheal intubation (6 cm of PE-240 polyethylene catheter), the common bile duct was cannulated (15 cm of PE-20 polyethylene tubing) after ligation of duodenal end. The rats were allowed to stabilize for a period of 2 h before challenge with OVA. Bile was collected for 1 h before and for eight consecutive periods of 1 h after OVA challenge. All bile samples were collected on ice in 1.5-ml Eppendorf tubes under a stream of argon. The bile was kept frozen at −80°C before analysis.

Procedures for antigen challenge and measurement of pulmonary mechanics. Rt was measured during tidal breathing. A Fleisch no. 0 pneumotachograph coupled to a differential transducer (Micro-Switch 163PC01D36, Honeywell, Scarborough, Ontario) was attached to a Plexiglas box into which was inserted the top of the endotracheal tube (6 cm of PE-240 polyethylene tubing) to measure airflow. Changes in esophageal pressure were measured by using a saline-filled catheter and a differential pressure transducer (Sanborn 267 BC, Hewlett-Packard, Waltham, MA). The other port of the transducer was connected to the Plexiglas box. The esophageal catheter consisted of 20 cm of PE-200 polyethylene tubing attached to a shorter 6-cm length of PE-100 tubing that was advanced into the esophagus of the rat until a clear cardiac artifact was discernible. Transpulmonary pressure was computed as the difference between esophageal and box pressures. Airway responses were evaluated from Rt, which was determined by fitting the equation of motion of lung by multiple linear regression using commercial software (RHT Infodat, Montreal, Quebec). The measurements were performed every 15 min for 8 h after the OVA challenge. The aerosols of saline, budesonide, and OVA were administered into the Plexiglas chamber, from which the anesthetized animals breathed spontaneously.

Purification of N-acetyl-LTE₄ from bile by HPLC. Bile samples were thawed, and methanol was added to 0.3-mL aliquots to give a final concentration of 80% (28). After centrifugation, the supernatants were adjusted to a concentration of 30% methanol and a pH of 3 and subjected to precolumn extraction reverse-phase HPLC, as previously described (24). The mobile phase consisted of a mixture of 64% methanol in aqueous buffer (1 mM EDTA and 0.1% acetic acid, adjusted to a pH of 5.4 by the addition of ammonium hydroxide). The flow rate was 0.7 ml/min. Ultraviolet absorbance was monitored by a variable wavelength ultraviolet detector (model 481, Waters). The retention time of standard N-acetyl-LTE₄ using these conditions was 16 min.

Radioimmunoassay. Column fractions were evaporated to dryness in a centrifuge under vacuum, and the residues were dissolved in phosphate-buffered saline (0.1 ml at pH 8.2). N-acetyl-LTE₄ was measured in each fraction by using a monoclonal antibody directed against LTC₄, which cross-reacted with N-acetyl-LTE₄. [14,15-³H]LTC₄ was used as the radioactive ligand.

Measurement of cells in BAL fluid. BAL was performed via a tracheal cannula with 5 × 5 ml of sterile Ca²⁺/Mg²⁺-free Hanks’ balanced salt solution at 37°C. The fluid was recovered through a disposable syringe and immediately centrifuged at 250 g for 10 min. After they were washed twice, the cells were resuspended in Hanks’ balanced salt solution and counted in a hemacytometer to determine total cell numbers. Differential cell counts were determined by an observer blinded as to the experimental group. A total of 200 cells were counted on each cytospin slide, which was stained with a modified May-Grunwald Giemsa stain.

Statistical analysis. Comparison of means was performed by using unpaired t-tests. Correlation coefficients were determined by using the Pearson product-moment correlation. Differences were considered to be statistically significant when P values were <0.05.
RESULTS

Effects of inhaled budesonide on airway resistance in response to antigen challenge. Figure 2A shows the time course for changes in airway resistance after antigen challenge in both control and budesonide-treated BN rats. The value of R\textsubscript{L} for the budesonide-treated animals was lower than that for the control animals at all time points investigated. However, there was considerable variability in the magnitude and timing of the late response among animals in the control group, resulting in large standard errors, especially at the later time points.

The effects of inhaled budesonide on the early and late airway responses are shown in Fig. 3A. The magnitude of the early response was expressed as the maximal increase in R\textsubscript{L} occurring within the first 30 min after antigen challenge. Budesonide appeared to reduce the magnitude of the early response by \( \sim 50\% \), but, because of the variability in the responses of individual animals, this difference was not statistically significant (\( P = 0.12 \)). This was also true when the early response was expressed as the area under the curve (AUC) between either 0 and 30 min (\( P = 0.21 \)) or 0 and 60 min (\( P = 0.17 \)) (data not shown).

The magnitude of the late response was determined from measurements made between 3 and 8 h after antigen challenge. It was calculated from the AUC for R\textsubscript{L} during the late response. The values for biliary N-acetyl-LTE\textsubscript{4} during the early response are the excretion rates for the 1st hour after antigen challenge, whereas those for the late response are the excretion rates between 3 and 8 h after antigen challenge. All values are means \( \pm \) SE (\( n = 6 \) rats). \( *P < 0.02 \) and \( **P < 0.005 \), compared with control for the same time period.

Effect of budesonide on the excretion of N-acetyl-LTE\textsubscript{4} in bile in OVA-challenged rats. The time courses for the effects of OVA on the excretion of N-acetyl-LTE\textsubscript{4} in the bile of budesonide-treated and control rats are shown in Fig. 2B. In the control rats, the levels of
increase in the levels of 0.6). In contrast, budesonide completely eliminated the stance during the last 5 h of the experiment. Budesonide had only a relatively small effect on the levels of \(N\)-acetyl-LTE\(_4\) but strongly inhibited the excretion of this sub-

The numbers of neutrophils in the BAL fluid also was 2.4 \(6\) \(\times 10^6\) cells in the control rats; 0.66 \(6\) \(\times 10^6\) in the budesonide-treated group \(P < 0.01\). This difference could be attributed primarily to a reduction in the numbers of macrophages in BAL fluid from the budesonide-treated rats \(0.35 \pm 0.04\) vs. 1.47 \(\pm 0.28 \times 10^6\) cells in the control rats \(P < 0.005\). The numbers of neutrophils in the BAL fluid also appeared to be lower in the budesonide-treated group, but there was a high degree of variability among the controls, and this difference was not statistically significant. Three animals in the control group exhibited lower neutrophil counts than the mean for the budesonide-treated group, whereas the remaining three animals displayed substantially higher neutrophil counts than any of those treated with budesonide.

Relationships between the late response, LT production, and BAL macrophages. When the data for individual rats were plotted, a positive correlation was observed between the magnitude of the late response and the excretion rate of \(N\)-acetyl-LTE\(_4\) in the bile (Fig. 5). Of the six animals in the control group, five exhibited a late response, whereas one animal exhibited no increase above baseline in the AUC for \(R\). When the data from all animals were considered, a correlation coefficient of 0.58 \(P < 0.05\) was obtained. This increased to 0.92 \(P < 0.0001\) when the data from the rat lacking a late response were excluded.

There was also a positive correlation between the excretion of \(N\)-acetyl-LTE\(_4\) in bile and the number of macrophages in BAL fluid from each of the rats (Fig. 6A). The correlation coefficient for this relationship was 0.80 \(P < 0.002\). However, it must be borne in mind that this correlation was dependent on the uniformly low numbers of macrophages in the budesonide-treated group and that other factors may also be involved in regulating LT synthesis. The magnitude of the late response also appeared to be positively correlated with the numbers of macrophages recovered in BAL fluid \((r = 0.63, P < 0.05;\) Fig. 6B). Exclusion of the data from the single rat that lacked a late response increased the correlation coefficient to 0.83 \(P < 0.002\). Neither biliary \(N\)-acetyl-LTE\(_4\) levels nor the magnitude of the late response was found to be correlated with the numbers of any of the other types of leukocyte detected in BAL fluid (data not shown).
levels of biliary rats exhibiting early responses displayed much higher controls. Interestingly, the two budesonide-treated played early responses less than those of any of the control rats, whereas the remaining four rats all displayed a significant, principally because two of the six animals in BAL fluid. 8 h after OVA challenge and the number of macrophages recovered in BAL fluid. Solid lines, regression lines for all the data calculated as described in Fig. 3 legend, and the number of macrophages recovered in BAL fluid. Dashed line, regression line for the data excluding the outlier.

DISCUSSION

We previously showed that systemic treatment of BN rats with dexamethasone (300 μg/kg ip 14 and 2 h before antigen challenge) significantly inhibited (by 60%) the early response to antigen challenge and completely eliminated the late response along with the associated increase in biliary cys-LTs (28). In the present study, we investigated the effects of topical budesonide on these responses, as well as cellular infiltration during the first 8 h after OVA challenge. Budesonide clearly blocked the late response, but we were unable to demonstrate an effect on the early response. Although the magnitude of the early response was reduced by ~50%, this effect was not significant, principally because two of the six animals in this group (data not shown). However, there was no overall correlation between biliary N-acetyl-LTE4 levels and the magnitude of the early response in the animals studied, nor did budesonide affect the levels of this substance during this period. The BN rat model used in these experiments has been developed to examine the late response, and the OVA pretreatment protocol employed before OVA challenge is not optimal for observation of the early response. The rather modest early responses observed in these animals may have made it difficult to detect modest changes in airway resistance at this time.

In the present study, we found that topical treatment with the glucocorticoid budesonide completely eliminated the late response to antigen, consistent with other reports in the literature (1, 7), as well as with our earlier study utilizing intraperitoneal dexamethasone (28). In addition to its effects on airway responses, budesonide strongly inhibited antigen-induced cys-LT synthesis in vivo. In contrast to our previous study (28), in which we found that dexamethasone eliminated both the immediate and later increases in cys-LT production in response to antigen, topical treatment of BN rats with budesonide over a similar time period appeared to have a somewhat delayed effect on cys-LT synthesis, which was not apparent until the fourth hour after challenge. It is possible that this may be due to differences in the effectiveness of the doses we used for the two glucocorticoids, or alternatively that the enhanced initial response to dexamethasone may be due to its systemic administration.

In contrast to our studies in the BN rat, it has not been possible to demonstrate an inhibitory effect of glucocorticoids on the in vivo synthesis of cys-LTs by either healthy human subjects (22, 34) or atopic asthmatic patients (26) or by antigen-challenged guinea pigs (15). However, the ex vivo production of LTB4 by BAL cells was suppressed by systemic prednisone in human subjects (34). It is thus possible that the effects of glucocorticoids on cys-LT synthesis are cell specific, making it difficult to observe effects on the in vivo production of these substances, which could be synthesized by a variety of cell types. The responsiveness of antigen-induced cys-LT synthesis in the BN rat to glucocorticoids may reflect different sites for the synthesis of these substances in this species, as discussed below. Moreover, the rat model offers the advantage that the bile duct can be cannulated, enabling the bile, which is the major route for the excretion of cys-LTs (10, 17), to be collected continuously throughout the experiment. This may make it possible to detect increased pulmonary release of cys-LTs that would be masked by background levels released from other sites when urine is collected over longer time periods.

Consistent with previous findings (24, 28), there was a positive correlation between the magnitude of the late response in BN rats and the levels of N-acetyl-LTE4 in the bile (r = 0.58; Fig. 5). However, one of the rats in the control group did not exhibit a late response, which is not surprising, in view of our previous findings.
that this response does not occur in ~30% of BN rats (12). The correlation coefficient for this relationship increased dramatically to 0.92 when the data from this animal were excluded from the analysis. In contrast to previous studies (24), this rat displayed relatively high levels of cys-LT production, despite the lack of a late response. It also exhibited the second highest levels of BAL macrophages and the second most intense early response of the animals in this group but could not be considered to be an outlier on the basis of any of the other responses investigated. The reason for the lack of a late response in this animal is, therefore, unclear but could conceivably be related to reduced responsiveness to cys-LTs. Despite the rats being highly inbred, variability in OVA-induced late responses in BN rats has been previously observed and appears to be related to differences in lymphocyte proliferation after stimulation with OVA (40).

Budesonide significantly reduced the numbers of macrophages in BAL fluid 8 h after challenge but had little effect on the numbers of lymphocytes and eosinophils and only a marginal effect on neutrophils. However, only small numbers of eosinophils were observed in BAL fluid 8 h after OVA challenge, in agreement with our earlier findings (44) showing that eosinophil numbers take ~24 h to reach high levels in the rat. It was not possible to prolong these experiments for more than 8 h because of the requirement for tracheal intubation and cannulation of the bile duct. It should be noted that, because of the predominance of macrophages in BAL cells, budesonide did not significantly reduce the percentage of macrophages in BAL (66.5 ± 9.0% in OVA controls vs. 53.6 ± 6.3% in the budesonide-treated group; \( P > 0.05 \)). Inhaled budesonide has previously been noted to result in reduced numbers of pulmonary macrophages with the phenotype of antigen-presenting cells in a long-term (3-mo) study in asthmatic subjects (4). The mechanism for the effect of budesonide on BAL macrophage levels is not clear but could possibly be related to a reduction in monocyte infiltration due to reduced levels of intercellular adhesion molecule-1 on endothelial cells (6). An effect on bone marrow progenitor cells is another possibility. Inhaled budesonide has been shown to block the increase in granulocyte-macrophage progenitor cells in bone marrow in dogs after antigen challenge (43).

Inhaled (30) or systemic (13, 30) glucocorticoids have also been shown to block the antigen-induced increase in BAL fluid eosinophils in BN rats. However, in these studies, inflammatory cells were measured 18–24 h after antigen administration, in contrast to the present study in which these cells were examined 8 h after challenge, immediately after the last measurement of Rt... Our laboratory previously found that dexamethasone (ip) did reduce the numbers of eosinophils in large and small airways of BN rats 8 h after OVA challenge. However, antigen-induced eosinophilia is maximal in these animals between 24 and 32 h (29, 44), and the increase in lung tissue may not be reflected in BAL fluid by 8 h.

The levels of N-acetyl-LTE\(_4\) in the bile of BN rats during the late response appeared to be positively correlated with the numbers of macrophages present in BAL. The macrophage may be an important site for cys-LT production in the rat (44). In contrast to human eosinophils, rat eosinophils do not produce appreciable amounts of these products (44), and mast cells, another major site of cys-LT production in humans (21), are much less abundant in rat lungs (44). Whether the reduction in cys-LT production induced by budesonide is due primarily to a reduction in macrophage numbers, or whether this substance also directly inhibits the production of cys-LTs, for example, by downregulating cytosolic, calcium-dependent phospholipase A\(_2\) (20), was not addressed in the present study.

In conclusion, inhaled budesonide abolished the antigen-induced late response and the associated increase in cys-LT synthesis in BN rats, possibly due to a reduction in the levels of pulmonary macrophages. Although the present study does not establish a causal relationship, it would seem likely that the reduction in LT synthesis would contribute to the effect of budesonide on airway responses. In addition, it is possible that budesonide could reduce the responsiveness of the airways to cys-LTs, as our laboratory previously showed for dexamethasone (28).

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Present address of L. Xu: Merck Frost Centre for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada H9R 4P8.

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